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ir. An CERDOBBEL

ENGINEERING THE THERMOSTABILITY OF SUCROSE PHOSPHORYLASE FOR INDUSTRIAL APPLICATIONS

Thesis submitted in fulfilment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:
Engineering van de thermostabiliteit van sucrose phosphorylase voor industriële toepassingen

Cover illustration: "Three-dimensional structure of sucrose phosphorylase colored by B-factor."

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An, 2 januari 2011

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ABBREVIATIONS

For terminology of nucleotides and amino acids see appendices I-III.

ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
*Ba*SP	Sucrose phosphorylase from *B. adolescentis*
BCA	Bicinchoninic acid
BCCM	Belgian Co-ordinated Collections of Micro-organisms
B-FIT	B-factor iterative test
BSA	Bovine serum albumin
CAZy	Carbohydrate active enzyme database
CD	Circular dichroism
CLEA	Cross-linked enzyme aggregate
CLEC	Cross-linked enzyme crystal
CV	Coefficient of variance (=standard deviation/mean × 100 %)
DSC	Differential scanning calorimetry
EC	Enzyme commission
EC-EP	Enzyme carrier epoxide
EC-HFA	Enzyme carrier hetero-functional amino
EDTA	Ethylenediaminetetraacetic acid
epPCR	Error-prone PCR
Fru	Fructose
G6P-DH	Glucose-6-phosphate dehydrogenase
GH	Glycoside hydrolase
Glc	Glucose
GP	Glycoside phosphorylase
GT	Glycosyl transferase
HTS	High-throughput screening
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	1000 base pairs
k_{cat}	Catalytic constant
kDa	KiloDalton
K_{eq}	Equilibrium constant
K_m	Michaelis-Menten constant

LB	Luria-Bertani broth
*Lm*SP	Sucrose phosphorylase from *L. mesenteroides*
MTP	Microtiter plate
NAD	Nicotinamide adenine dinucleotide
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
PB	Permeabilization buffer
PCR	Polymerase chain reaction
PDB	Protein data bank
PGM	Phosphoglucomutase
PMSF	Phenylmethylsulfonyl fluoride
RMSD	Root mean square deviation
SN	Supernatant
SP	Sucrose phosphorylase
SU	Suspension
$t_{1/2}$	Half-life time
T_{50}	The temperature at which half of the activity is lost
TG	Transglucosidase
T_m	Melting temperature
T_{opt}	Optimal temperature
Tris	Tris(hydroxymethyl)aminomethane
U	Unit of enzymatic activity
UDP	Uridine diphosphate
αG1P	α-D-glucose-1-phosphate

INTRODUCTION

INTRODUCTION

The synthesis of glycosidic bonds is of high commercial value, because the produced compounds can be used for a wide range of applications. Oligosaccharides, for example, have received a lot of attention in recent years for their potential prebiotic effects in food preparations (Roberfroid, 2007). Such molecules should not be hydrolysed by digestive enzymes and stimulate the growth of beneficial bacteria (mainly *Bifidobacteria)* in the intestines. Fructo-oligosaccharides (FOS) probably are the most famous example but other prebiotics that are produced by enzymatic glycosyl transfer include galacto-oligosaccharides (GOS) and isomalto-oligosaccharides (IMO) (Hirayama, 2002). In addition, oligosaccharides can also serve as low-caloric, non-cariogenic sweeteners that are healthy alternatives to the classical table sugar sucrose.

Attaching a glycosyl group to a non-carbohydrate acceptor can drastically change both the physicochemical and biological properties of that molecule. Glycosylation can thus be used to increase the solubility of hydrophobic compounds, to improve the pharmacodynamics of drugs, to optimize the activity spectrum of antibiotics, and to modulate flavours and fragrances. This is nicely illustrated by flavonoids, as their pharmaceutical properties can often be efficiently exploited only in the form of their hydrophilic glycosyl derivatives (Kren, 2008). Another famous example is ascorbic acid, a very sensitive vitamin whose long-term storage can be drastically increased by glucosylation (Yamamoto *et al.*, 1990). The glucoside of menthol, in turn, can function as a controlled release compound, being slowly hydrolysed in the mouth to prolong the sensation of freshness (Nakagawa *et al.*, 1998).

Since the days of Emil Fischer, a lot of new developments have been realized in the chemical synthesis of glycosides (Fischer, 1893; Nicolaou & Mitchell, 2001). Nevertheless, the selective formation of a glycosidic bond still is a challenging task. It requires the use of protecting and activating groups, resulting in multi-step synthetic routes with a low overall yield. Furthermore, toxic catalysts (heavy metals) are often involved and large amounts of waste are generated. Enzymes are a very attractive alternative as they are highly specific and are active under mild reaction conditions. It has been calculated that enzymatic glycosylation reactions generate 5-fold less waste and have a 15-fold higher space-time yield, a tremendous improvement in eco-efficiency (de Roode *et al.*, 2003).

Many enzymes can be applied for the production of glycosides. We have selected sucrose phosphorylase (SP) because it can transfer a glucosyl moiety from an inexpensive donor substrate -simple table sugar- to a wide variety of acceptor molecules (CHAPTER 1).

Unfortunately, the thermostability of this enzyme is too low for industrial applications, which need to be operated at 60 °C or higher to avoid microbial contamination. In this thesis, various strategies will be evaluated to improve the performance of sucrose phosphorylase at high temperatures. First, the most promising SP enzymes will be recombinantly expressed and thoroughly characterized to identify the best template for engineering experiments (CHAPTER 2). The determinants of SP thermostability will then be examined by sequence alignments and mutational analysis (CHAPTER 3). In a next step, both directed evolution (CHAPTER 4) and (semi-)rational design (CHAPTER 5) will be used for the creation of enzyme variants with enhanced stability. In parallel, rigidification of the enzyme's structure will be attempted by multipoint covalent immobilization (CHAPTER 6) and by crosslinking of the enzyme (CHAPTER 7). Finally, various production processes will be developed with the obtained biocatalysts to provide proof of concept of their potential industrial applications (CHAPTER 8).

In summary, the goal of this thesis is to turn sucrose phosphorylase into a suitable biocatalyst for the production of glycosides at the industrial scale. Before the results are discussed, an overview of the available literature will be presented to provide the reader with sufficient background information.

CHAPTER 1

LITERATURE REVIEW

1. CAZYMES

1.1 Introduction

In terms of quantity, the transfer of a glycosyl group is one of the most important biochemical reactions in nature. Indeed, glycosidic molecules represent about two thirds of the carbon in the biosphere, largely in the form of (hemi)cellulose and chitin (Stern & Jedrzejas, 2008). These β-linked polysaccharides provide structural support, while α-linked glycans such as starch and glycogen function as energy storage. Oligosaccharides, in turn, can be essential nutrients that stimulate the immune system (e.g. human milk oligosaccharides) or have transport (e.g. raffinose series) and signalling (e.g. oligosaccharins) functions in plants (Lehman, 1998). Finally, glycosidic bonds are also present in a wide range of glycosides and glycoconjugates, in which the glycon part often is crucial for biological activity (Varki, 1993; Kren, 2008). The synthesis as well as the degradation of all of these molecules involves the enzymatic transfer of glycosyl groups.

Due to the presence of multiple hydroxyl groups that can be used as point of attachment, saccharides display much more variability than other biopolymers, *i.e.* proteins and nucleic acids. From glucose, for example, 11 different disaccharides can be synthesized, while only 1 homodimer can be produced from either alanine or adenine (Laine, 1994). Consequently, the processing of glycosidic molecules involves a wide range of enzymes, which typically account for 1-3% of an organism's genes (Coutinho *et al.*, 2003). A glycosidic bond is also more stable than a phosphodiester or peptide bond, with a half-life at room temperature in the order of 10^7 years ($k_{uncat} = 10^{-15}$ s^{-1}) (Wolfenden *et al.*, 1998). Its degradation thus requires enzymes that are exceptionally proficient. Glycoside hydrolases can achieve rate enhancements up to 10^{17}, a number almost unparalleled in nature.

Enzymes that display activity towards glycosidic bonds are collectively known as carbohydrate-active enzymes (CAZymes). Their use in hydrolytic reactions (transfer to water) has already been exploited at a large scale for a very long time -mainly in the starch industry- and will not be treated here (Crabb & Mitchinson, 1997; Kelly *et al.*, 2009). In contrast, this chapter will focus on the synthetic applications of CAZymes, comparing the advantages and disadvantages of each enzyme class. Although polysaccharide lyases are also part of the CAZyme family, they do not catalyze the transfer of a glycosyl group and will thus not be included in the discussion (Michaud *et al.*, 2003).

1.2 Classification

There are basically four different types of enzymes that can catalyze the transfer of a glycosyl group (Figure 1.1) (Lairson & Withers, 2004). The vast majority of glycosylation reactions in nature are performed by glycosyl transferases (GTs) that use nucleotide-activated sugars as glycosyl donor. Transglycosidases (TGs), in turn, are able to synthesize saccharides by transferring a glycosyl group from one carbohydrate chain to another. In contrast, glycoside hydrolases (GHs) and glycoside phosphorylases (GPs) catalyze the degradation of glycosidic bonds, using water and inorganic phosphate, respectively, as the acceptor substrate.

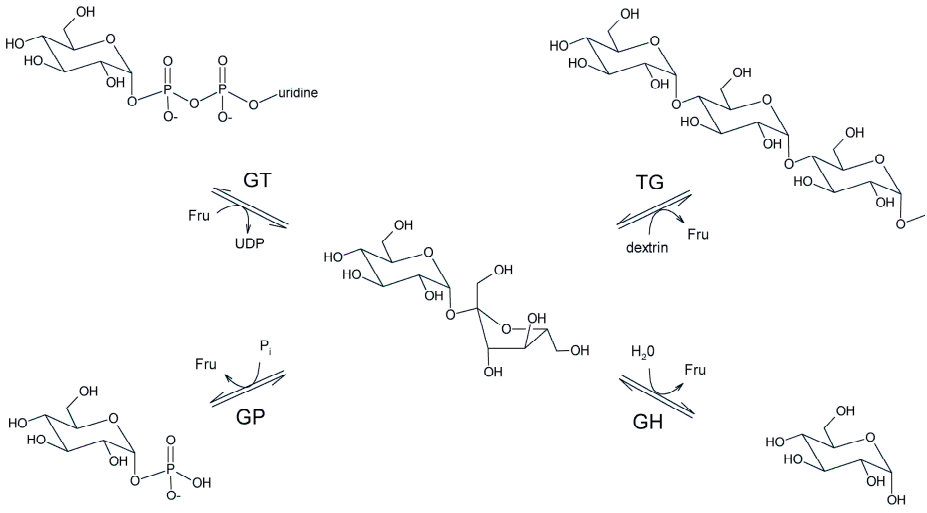

Figure 1.1 The four different types of CAZymes illustrated by their respective reactions with sucrose. Sucrose is synthesized by sucrose synthase (GT) and can be converted into amylase by amylosucrase (TG) or degraded by either sucrose phosphorylase (GP) or sucrose hydrolase (GH).

These enzymes can be classified in different ways, depending on what criterion is used (Table 1.1). The most widely used classification system undoubtedly is that of the Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB) (Webb, 1992). It is based on the specificity of the reaction and unambiguously defines the substrates and products involved. In that system, CAZymes that use water as the acceptor substrate are classified as glycoside hydrolases (EC 3.2), while all others are classified as glycosyl transferases (EC 2.4). It is then, perhaps, not surprising that the latter subclass comprises more specificities (~ 300) than the former (~ 200).

Table 1.1 Different classification schemes for CAZymes

Enzyme type	Metabolic role	EC-subclass	CAZy family
GH	degradation	3.2	GH
GP	degradation	2.4	GH/GT
TG	synthesis	2.4	GH
GT	synthesis	2.4	GT

Although not without its merits, the EC-classification is somewhat arbitrary because all CAZymes catalyze a formal transfer reaction, whatever the acceptor substrate may be. By separating glycosidases from the other enzymes, their functional and mechanistic relationships become obscured. Indeed, the inorganic phosphate used as nucleophile by GPs is not that different from water, while TGs are nothing more than specialized retaining glycosidases. The EC-classification also creates the false impression that all glycosyl transferases are involved in synthetic reactions, while this is clearly not the case for GPs. To discriminate the 'true' glycosyl transferases from the other enzymes in subclass 2.4, the term Leloir transferases is often used (see section 1.6).

The classification of enzymes based on sequence alignments is a very powerful alternative because it reveals their evolutionary relationships (Sammut *et al.*, 2008). To that end, related enzymes (sequence identity of about 30 % or higher) are grouped in so-called families in which the structural fold as well as the reaction mechanism is conserved. General features that have been elucidated for one family member can thus easily be transferred to another, and this predictive power is one of the main advantages of sequence-based classifications. Substrate specificity, however, is not a conserved feature as this is dictated by subtle differences in the enzymes' active sites. A single family thus often contains several EC-entries, although the substrates usually have the same anomeric configuration (e.g. β-glucosidase and β-galactosidase). Furthermore, one EC-entry can sometimes be found in more than one family, if nature has evolved more than one structural template and/or catalytic mechanism to perform the same reaction.

Such a classification scheme for CAZymes was developed by Henrissat and co-workers almost two decades ago (Henrissat, 1991). They have since established a database that is freely accessible online and is simply known as CAZy (Cantarel *et al.*, 2009). This curated and regularly updated database has proven to be an essential tool in the study of the mechanism and structure of CAZymes. These enzymes were found to fall in two broad categories, *i.e.* either GH- or GT-sequences, both of which now comprise about 100 families. TGs belong to GH-families,

which reflects their mechanistic analogy with retaining glycosidases. GPs, however, can be found in both GH- and GT-families, revealing their multiple evolutionary origins (see section 1.7). This illustrates that the unambiguous classification of CAZymes is not trivial and can be confusing for the non-expert reader.

1.3 Reaction mechanisms

Glycoside hydrolases have been studied most extensively and will be used as case study to illustrate single and double displacement mechanisms. GHs hydrolyze their substrate with either inversion or retention of the anomeric configuration. General mechanisms for these reactions were already proposed more than five decades ago by Daniel Koshland (Koshland, 1953) and have since been corroborated by a wealth of structural and kinetic data (Sinnott, 1994; Davies & Henrissat, 1995; Rye & Withers, 2000; Vasella *et al.*, 2002; Vocadlo & Davies, 2008). Two carboxylic amino acids (Glu or Asp) play a crucial role in both mechanisms, albeit with slightly different functions (Figure 1.2).

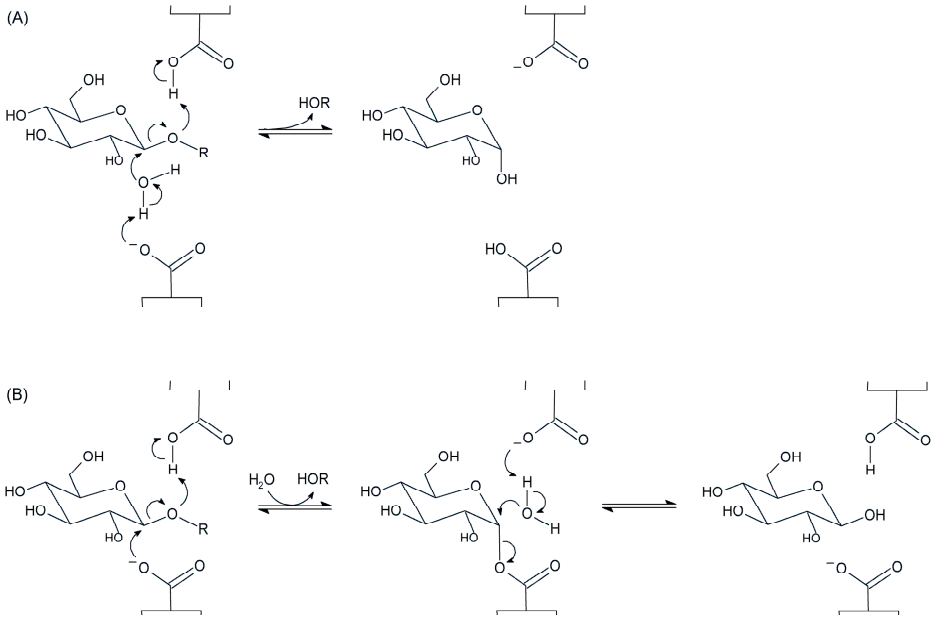

Figure 1.2 Reaction mechanism of β-glycosidases. A. Inverting glycosidases follow a single displacement mechanism B. Retaining glycosidases follow a double displacement mechanism.

Inverting glycosidases follow a single displacement mechanism, catalyzing a direct nucleophilic attack of water on the anomeric carbon. One carboxylic residue (the catalytic base) assists the water molecule by accepting a proton, while the other residue (the catalytic acid) activates the leaving group by donating a proton. In the end, both catalytic amino acids have to exchange protons with the solvent to revert to their original ionization state and allow a new reaction to take place. This mechanism is characterized by the formation of a ternary complex, in which both a saccharide and a water molecule are simultaneously present in the enzyme's active site. As the water molecule is positioned in between the catalytic base and the anomeric carbon, the distance between the catalytic amino acids is considerable larger (~10 Å) than that in retaining glycosidases (~5.5 Å) (Zechel & Withers, 2000).

Retaining glycosidases follow a double displacement mechanism that is characterized by the formation of a covalent glycosyl-enzyme intermediate. One carboxylic residue (the catalytic nucleophile) now attacks the anomeric carbon first, resulting in an ester bond that can be hydrolyzed more efficiently than the original ether bond in the substrate. Consequently, the formation of the covalent intermediate nearly always is the rate-limiting step of the overall reaction sequence. The other residue (the catalytic acid/base) protonates the leaving group in the first step and subsequently deprotonates water in the second step. The double inversion of the anomeric configuration results in a net retention. Although the covalent intermediate has a very short lifetime, it can be studied by X-ray crystallography with a special trapping strategy developed by Withers and co-workers (Withers, 1999).

For retaining GTs, however, an internal replacement mechanism has been proposed as an alternative to the classical double displacement mechanism of retaining glycosidases (Persson *et al.*, 2001). As conserved catalytic residues or a covalent intermediate seem to be absent in these enzymes, a ternary complex is most likely formed in which the phosphate moiety of the donor substrate deprotonates the acceptor molecule (Figure 1.3). The latter then performs a nucleophilic attack onto the oxocarbenium intermediate from the front side, resulting in retention of the anomeric configuration. Inverting GTs employ a reaction mechanism that is similar to that of inverting hydrolases, albeit with one instead of two catalytic residues (Lairson *et al.*, 2008). Indeed, the nucleotide group does not need to be protonated for departure, eliminating the need for a catalytic acid.

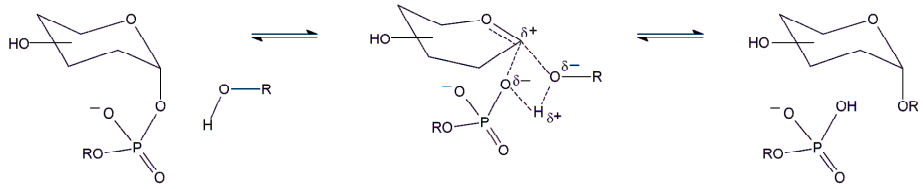

Figure 1.3 Proposed mechanism of retaining GTs. The departing phosphate group deprotonates the acceptor molecule, which then performs a nucleophilic attack onto the oxocarbenium intermediate from the front side.

1.4 Glycoside hydrolases

Of all types of CAZymes, glycosidases have been studied most extensively (Davies & Henrissat, 1995). They hydrolyze their substrate with either inversion or retention of the anomeric configuration. Back in 1965, hen egg white lysozyme (HEWL) was the first enzyme to have its crystal structure determined and its mechanism has since become a textbook example of enzymatic chemistry (Blake *et al.*, 1965; Johnson & Phillips, 1965). Remarkably, however, proof of the occurrence of a covalent glycosyl-enzyme intermediate in the reaction of this retaining glycosidase has only recently been obtained (Vocadlo *et al.*, 2001). By now, nearly all GHs have been shown to follow the classical single and double displacement mechanisms, with few exceptions (Vocadlo & Davies, 2008).

Although glycosidases typically degrade their substrate in quantitative yields, they can also be used for synthetic purposes. Hydrolases are very attractive in that respect because they are readily available, are rather cheap and robust, comprise a wide range of donor specificities and often display activity towards a variety of carbohydrate and non-carbohydrate acceptors. Most synthetic applications have been described for β-glucosidase and β-galactosidase, although enzymes with a more unusual donor specificity are potentially also very interesting (Kren & Thiem, 1997; Bojarová & Kren, 2009). A disadvantage of GHs, however, is their low regioselectivity, meaning that a mixture of products is often formed when the acceptor contains more than one hydroxyl group. Another disadvantage is the relatively low product yields, although these can be improved by performing the reaction under either thermodynamic or kinetic control.

Under thermodynamic control, the equilibrium is shifted in the synthetic direction to achieve 'reverse hydrolysis' (Figure 1.4a). To that end, high substrate concentrations or media with a low water content need to be used. Although a minimal amount of water (~10 %) is usually required to remain active, the stability of glycosidases in the presence of organic solvents can be improved by immobilization or by using thermostable enzyme variants (van Rantwijk *et al.*,

1999). The synthesis of alkyl glycosides, which have important applications as detergents, is especially convenient as an alcohol can serve as both acceptor substrate and organic component of the medium (Garcia-Garibay *et al.*, 2000; Balogh *et al.*, 2004). For the synthesis of oligosaccharides, in contrast, high substrate concentrations (up to 80% w/w) in aqueous media are typically used. In fact, the very first enzyme-catalyzed synthesis was performed in this way, *i.e.* the production of isomaltose from a concentrated glucose syrup by yeast extract (α-glucosidase) in 1898 (Sumner & Somers, 1953).

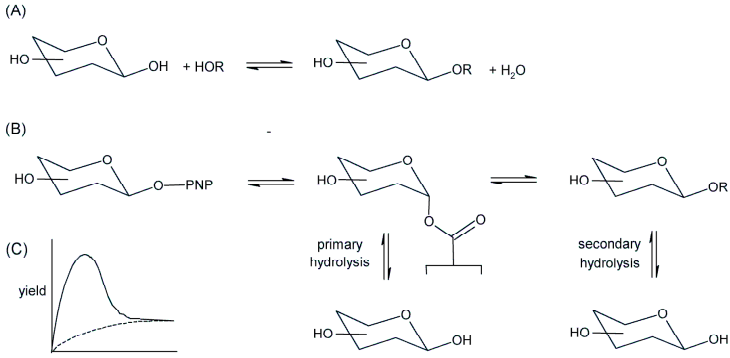

Figure 1.4 Synthetic reactions catalyzed by glycosidases. Yields obtained by kinetic control (B) are higher than those obtained by thermodynamic control (A) if the reaction is stopped before secondary hydrolysis takes over (C).

Kinetically controlled synthesis is a completely different concept that is based on the double displacement mechanism of retaining glycosidases. In that case, the covalent glycosyl-enzyme intermediate is intercepted by an acceptor substrate other than water, resulting in a transglycosylation reaction (Figure 1.4b). To maximize the yields, reaction conditions (pH, T) should be identified that result in the highest ratio of transglycosylation over hydrolysis, as these often differ from those for optimal activity (Eneyskaya *et al.*, 2009). Attention should also be paid to the fact that the glycosylated product can, in turn, serve as substrate and will eventually be degraded through secondary hydrolysis. Therefore, activated donor substrates such as *p*-nitrophenol (PNP) glycosides are preferred because these increase the ratio of synthesis over secondary hydrolysis (van Rantwijk *et al.*, 1999). In that way, yields can be obtained that 'overshoot' the equilibrium conversion of the reactants, at least if the reaction is stopped before thermodynamic control takes over (Figure 1.4c). Using PNP β-galactoside as donor, for example, a yield of nearly 40% could be obtained for the synthesis of *N*-acetyllactosamine, a core component of the glycan structure of glycoproteins (Hernaiz & Crout, 2000). In contrast, a yield

of only 20% is obtained for the glucosylation of caffeic acid with maltopentaose as donor (Nishimura *et al.*, 1995).

One of the major advances in the use of GHs for synthetic applications, has been the development of so-called glycosynthases (Mackenzie *et al.*, 1998). These are mutant glycosidases that are devoid of hydrolytic activity but can still perform transglycosylation reactions when a suitable activated donor is presented (Perugino *et al.*, 2005; Shaikh & Withers, 2008). More specifically, the catalytic nucleophile of a retaining glycosidase is mutated and a glycosyl fluoride with the opposite anomeric configuration is used as donor (Figure 1.5). A fluor atom is sufficiently small to access the space generated by the removal of the nucleophile, mimicking the configuration of the covalent intermediate for attack by an acceptor substrate. Recently, the glycosynthase concept has been extended to inverting glycosidases, albeit with a different mutational strategy (Honda & Kitaoka, 2006; Honda *et al.*, 2008). Furthermore, mutant hydrolases known as thioglycosynthases and thioglycoligases have been created for the synthesis of glycosidic bonds that contain sulphur instead of oxygen as heteroatom (Jahn *et al.*, 2003; Jahn *et al.*, 2004).

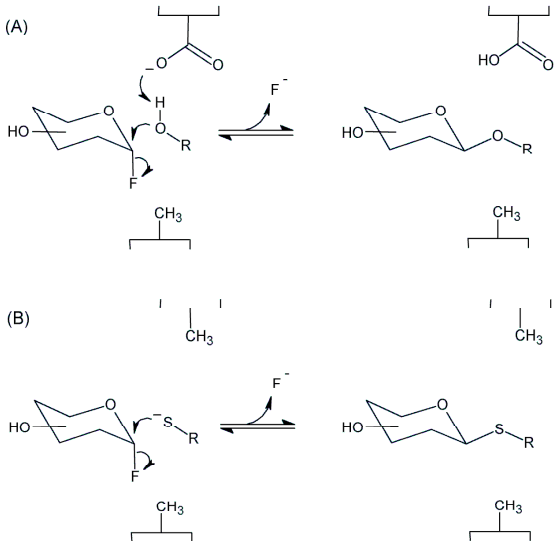

Figure 1.5 Mechanism of glycosynthases. A. Inactivation of the nucleophile of glycosidases destroys the hydrolytic activity but still allows the transfer of a glycosyl group from a fluoride donor with the wrong anomeric configuration. B. Addidional inactivation of the catalytic acid/base allows the synthesis of thioglycosidic bonds, as thiols do not need to be activated by deprotonation for a nucleophilic attack.

In principle, glycosynthases can be developed for all GH specificities and offer an unlimited array of enzymes to catalyze glycosylation reactions with very high yields (> 90%). This has already been exploited for several GHs with important donor specificities. Glycosynthase variants have, for example, been created of α-L-fucosidase and β-glucuronidase to synthesize oligosaccharide structures of considerable biological significance (Mullegger *et al.*, 2006; Wada *et al.*, 2008). Other types of linkages that have become accessible include the β-mannoside bond, which is one of the most difficult to synthesize chemically (Nashiru *et al.*, 2001). For some enzymes, the regioselectivity of the produced glycosidic bond was found to depend on the type of acceptor substrate that is used (Faijes *et al.*, 2006; Blanchard *et al.*, 2007).

The acceptor specificity of glycosynthases is primarily determined by that of the parent enzyme. The glycosynthase derived from endoglycoceramidase II, for example, evidently displays activity towards a lipid acceptor (Vaughan *et al.*, 2006). Its ability to transfer sialyllactose to sphingosine is, however, a new activity for which a GT is not available, and has allowed the production of a ganglioside with important signalling functions. In addition, the mutant enzymes have sometimes been found to display activity towards non-natural acceptors as well. The glycosynthase derived from endoglucanase Cel7B, for example, can efficiently glycosylate flavonoids, an unexpected feature that has been exploited for the synthesis of therapeutic compounds (Yang *et al.*, 2007).

1.5 Transglycosidases

Transglycosidases (TGs) catalyse the conversion of one carbohydrate chain into another. They are basically retaining glycosidases that are able to avoid water as acceptor in the deglycosylation step, and thus display a high degree of kinetic control (Seibel *et al.*, 2006). The difference between GHs and TGs is, however, not always clear-cut. Although the latter enzymes usually catalyze only a minor hydrolytic reaction (< 10%), mixed-typed activities are sometimes observed (Rose *et al.*, 2002). Furthermore, all known transglycosidases are classified in GH-families and are, therefore, not easily recognized as a separate enzyme class (Table 1.2). Nevertheless, TGs have been used for the efficient synthesis of glycosidic bonds from cheap carbohydrates (*i.e.* sucrose or amylose) as donor substrate.

TGs are very interesting biocatalysts but their applications are limited by the small number of available specificities (Table 1.2). However, it should theoretically be possible to convert any retaining glycosidase into a transglycosidase by means of enzyme engineering. Although the improvements in transfer activity are often moderate, some successful examples of this strategy

have been reported (Hansson *et al.*, 2001; Goyal *et al.*, 2002; Feng *et al.*, 2005; Hinz *et al.*, 2006; Damian-Almazo *et al.*, 2008; Placier *et al.*, 2009). Among the most impressive results are the creation of variants of β-galactosidase and of α-L-fucosidase that display a transfer/hydrolysis ratio of 9 and 6, respectively, resulting in substrate conversions with minimal interference from hydrolysis (Jorgensen *et al.*, 2001; Osanjo *et al.*, 2007). Further research in this field should allow to drastically expand the range of glycosylated products that can be generated by transglycosylation reactions from cheap donor substrates.

Table 1.2 Overview of all known transglycosidases

Family	M[a]	Enzyme	Synthesized bond	EC
GH-13	R	amylosucrase	Glc-α1,4-Glc	2.4.1.4
		glucan branching enzyme[c]	Glc-α1,6-Glc	2.4.1.18
		cyclodextrin glucanotransferase	Glc-α1,4-Glc	2.4.1.19
		disproportionating enzyme[c]	Glc-α1,4-Glc	2.4.1.25
GH-16	R	xyloglucan endotransglycosylase[c]	Glc-β1,4-Glc	2.4.1.207
GH-32	R	sucrose fructosyl transferase	Fru-β2,1-Fru	2.4.1.99
		fructan fructosyl transferase	Fru-β2,1-Fru	2.4.1.100
GH-68	R	inulosucrase	Fru-β2,1-Fru	2.4.1.9
		levansucrase	Fru-β2,6-Fru	2.4.1.10
GH-70	R	dextransucrase	Glc-α1,6-Glc	2.4.1.5
		alternansucrase	Glc-α1,3/6-Glc	2.4.1.140
		mutansucrase	Glc-α1,3-Glc	2.4.1.-
		reuteransucrase	Glc-α1,4-Glc	2.4.1.-
UC[b]	R	dextrin dextranase	Glc-α1,6-Glc	2.4.1.2
		oligoglucan branching enzyme	Glc-α1,6-Glc	2.4.1.24
		sucrose glucosyl transferase	Glc-α1,6-Glc	2.4.1.125
		glucan glucosyl transferase	Glc-α1,4-Glc	2.4.1.161
		aldose fructosyl transferase	Gly-α1,2β-Fru	2.4.1.162
		raffinose galactosyl transferase	Gal-α1,1-Fru	2.4.1.166

[a] Mechanism: retaining; [b] Unclassified; [c] Can be found in more than one family.

1.6 Glycosyl transferases

Glycosyl transferases (GTs) *stricto sensu* are enzymes that require nucleotide-activated donors for the synthesis of glycosidic bonds. These enzymes are often referred to as 'Leloir' transferases, in honour of the Argentinean scientist who received the Nobel Prize in Chemistry (1970) for the discovery of the first sugar nucleotide (UDP-glucose) and the elucidation of the corresponding metabolic pathways (Cardini *et al.*, 1950). GTs are responsible for the majority of glycosylation reactions in nature and comprise a wide range of specificities. Although very efficient, the industrial application of transferases is hampered by the high price of their nucleotide-activated donors. Consequently, they have mainly been used for the synthesis of oligosaccharides and glycosides with therapeutic properties.

Glycosyl transferases have long been perceived as being frustrating to work with because they are often membrane-bound and difficult to obtain. However, several specificities are now commercially available, of which galactosyl transferase (GalT) probably is the most famous one. The mammalian enzyme has been extensively studied for its use in the synthesis of *N*-acetyllactosamine, a key component of the glycan epitopes present on glycoproteins (Palcic, 1999). Another important specificity is sialyl transferase, which caps glycan structures with a *N*-acetylneuraminic acid to modulate their recognition processes (Weijers *et al.*, 2008). These and other GTs have now also been identified in micro-organisms, which greatly facilitates their large-scale production (Johnson, 1999). Indeed, microbial glycosyl transferases are typically soluble proteins that can be efficiently expressed in a convenient host like *E. coli*.

Microbial GTs have been found to have a more relaxed substrate specificity than their mammalian counterparts. Micro-organisms also contain a number of transferases that display activity towards non-carbohydrate acceptors such as polyketide and macrolide antibiotics (Erb *et al.*, 2009). In those cases, the transferred glycosyl group typically is a deoxygenated sugar like D-olivose, L-rhodinose, L-mycarose or L-digitoxose. The promiscuous nature of the microbial enzymes is illustrated by the fact that some of them are also able to glycosylate aromatics, coumarins and flavanols (Yang *et al.*, 2005). Furthermore, it has also been shown that the substrate range of transferases can be expanded by means of enzyme engineering (Luzhetskyy & Bechthold, 2008; Williams *et al.*, 2008). Directed evolution of the oleandomycin GT (OleD), for example, has been used to introduce activity on 12 different donors and 11 non-carbohydrate acceptors that are not used by the wild-type enzyme (Williams *et al.*, 2007). Interestingly, the resulting glycosidic bonds could contain oxygen, nitrogen as well as sulphur as hetero-atom.

For the glycosylation of non-carbohydrate acceptors, plants form by far the richest source of biocatalysts (Bowles *et al.*, 2005). Indeed, they produce a wide range of secondary metabolites

whose properties are often modulated by glycosylation. The substrate specificity of these GTs is not limited to plant metabolites but also includes mycotoxins (as a defence mechanism against moulds) and xenobiotics (as a means of detoxification). An interesting example of the potential of plant transferases for the production of glycosides, is the glucosylation of the flavonoid quercetin with enzymes from *Arabidopsis* (Lim *et al.*, 2004). Using a recombinant *E. coli* as a whole-cell biotransformation system, the process could be performed a the fermenter-scale to generate glucosides with medicinal properties. Engineering of plant enzymes has also resulted in enzyme variants with optimized or altered specificities (Wang, 2009).

The major disadvantage of GTs is the high price of their nucleotide-activated donors. Therefore, *in situ* regeneration systems have been developed that allow continuous recycling of the sugar donor. This not only reduces the cost of the donor substrate, but also prevents product inhibition. Also whole-cell based production systems have been developed using metabolically engineered bacteria and coupling of bacteria (Endo *et al.*, 1999; Chen *et al.*, 2002; Lee *et al.*, 2002; Zhang *et al.*, 2003).

Although GTs convert their substrates with near quantitative yields, their reactions have been found to be reversible (Zhang *et al.*, 2006). In that way, sugar nucleotides can be produced from glycosylated products. Some of the more exotic glycosyl donors are otherwise very difficult to obtain from natural sources or are very complicated to synthesize. Furthermore, the reversibility of the reactions can also be used to transfer the glycosyl group from one aglycon to another, somewhat reminiscent of the activity of TGs. This exchange mechanism allows the substrate specificity of the enzymes to be screened without the availability of either sugar nucleotides or deglycosylated aglycons.

1.7 Glycoside phosphorylases

Glycoside phosphorylases (GPs) catalyze the degradation of the glycosidic linkage in di- and oligosaccharides with the use of inorganic phosphate, resulting in the production of a glycosyl phosphate and a saccharide of reduced chain length (Kitaoka & Hayashi, 2002). The glycosyl phosphate can be metabolized through glycolysis without further activation by a kinase, because the phosphate group can be simply transferred from C1 to C6 by a phosphomutase (Knowles, 1980). The phosphorolytic degradation of saccharides is, therefore, more energy-efficient than their hydrolysis, and amounts to the saving of one molecule of ATP. The name ‘phosphorylase’ is a historic anomaly and the more accurate ‘phosphorolase’ is almost never used.

From a functional perspective, phosphorylases are very similar to hydrolases, differing only in their use of phosphate instead of water to break a glycosidic bond. This difference has, however, an important practical consequence because the high energy content of the produced glycosyl phosphate allows the reactions to be reversed and to be used for synthetic purposes *in vitro*. In that respect, GPs resemble glycosyl transferases that also employ a glycosyl phosphate as donor, albeit one that is considerably larger and substituted with a nucleotide group.

The dual nature of phosphorylases is also revealed by their sequence alignment, as they can be found in both GH- and GT-families (Table 1.3) (Kitaoka & Hayashi, 2002). This means that the enzymes have evolved along different evolutionary paths to catalyze the same type of reaction. On the one hand, the active site of a hydrolase must have been modified to allow the binding of phosphate instead of water. On the other hand, the nucleotide binding pocket of a transferase must have been modified to increase the affinity for a shortened glycosyl donor. Phosphorylases thus nicely bridge the gap between GH- and GT-specificities and their study should provide crucial insights in the evolution of the different types of CAZymes.

Table 1.3 Overview of all known glycoside phosphorylases

Family	M[a]	Substrate	Glycosidic bond	Donor	EC
GH-13	R	sucrose	Glc-α1,2β-Fru	α-Glc-1P	2.4.1.7
GH-65	I	maltose	Glc-α1,4-Glc	β-Glc-1P	2.4.1.8
		trehalose	Glc-α1,1α-Glc	β-Glc-1P	2.4.1.64
		kojibiose	Glc-α-1,2-Glc	β-Glc-1P	2.4.1.230
GH-94	I	cellobiose	Glc-β1,4-Glc	α-Glc-1P	2.4.1.20
		cellodextrin	Glc-β1,4-Glc	α-Glc-1P	2.4.1.49
		chitobiose	GlcNAc-β1,4-GlcNAc	α-GlcNAc-1P	2.4.1.-
GH-112	I	(ga)lacto-*N*-biose	Gal-β1,3-GlyNAc	α-Gal-1P	2.4.1.211
		Galactosylrhamnose	Gal-β1,4-L-Rha	α-Gal-1P	2.4.1.247
GT-4	R	trehalose	Glc-α1,1α-Glc	α-Glc-1P	2.4.1.231
GT-35	R	glycogen/starch	Glc-α1,4-Glc	α-Glc-1P	2.4.1.1
UC[b]	I	trehalose-6P	Glc-α1,1α-Glc-6P	β-Glc-1P	2.4.1.216
		laminaribiose	Glc-β1,3-Glc	α-Glc-1P	2.4.1.31
		laminaridextrin	Glc-β1,3-Glc	α-Glc-1P	2.4.1.30
		laminarin	Glc-β1,3-Glc	α-Glc-1P	2.4.1.97

[a] Mechanism: retaining (R) and inverting (I); [b] Unclassified.

The donor specificity of most phosphorylases is restricted to glucose-1-phosphate, thus limiting their activity to the transfer of a glucosyl moiety. The only known exceptions are chitobiose phosphorylase and the GPs from GH-112 that use α-*N*-acetylglucosamine-1-phosphate and α-galactose-1-phosphate, respectively, as glycosyl donor (Hidaka *et al.*, 2004; Hidaka *et al.*, 2009). Furthermore, the donor specificity of cellobiose phosphorylase has recently been expanded towards α-galactose-1-phosphate by means of directed evolution (De Groeve *et al.*, 2009a). The resulting enzyme variant is known as lactose phosphorylase, a specificity that has not yet been observed in nature. Production processes for various glycosyl phosphates with both wild-type and variant GPs have been reported (Weinhausel *et al.*, 1995; Goedl *et al.*, 2007; De Groeve *et al.*, 2009b).

The acceptor specificity of phosphorylases is typically more relaxed and comprises a wide range of mono-, di- and oligosaccharides. Some important examples include L-sorbose, L-fucose, L-arabinose, isomaltose, melibiose, gentiobiose, raffinose, nystose and isopanose (Aisaka *et al.*, 1996; Percy *et al.*, 1998; Chaen *et al.*, 1999; Aisaka *et al.*, 2000; Okada *et al.*, 2003). Kojibiose phosphorylase was even found to glucosylate a cyclic tetrasaccharide with potential applications as a carrier for drug delivery (Watanabe *et al.*, 2005). Furthermore, the engineering of the acceptor specificity of cellobiose phosphorylase has resulted in enzyme variants that are able to glucosylate both alkyl and aryl glucosides (De Groeve *et al.*, 2010a; De Groeve *et al.*, 2010b). Non-carbohydrate acceptors have also been reported for GPs. In that respect, sucrose phosphorylase (SP) probably is the most interesting biocatalyst (Goedl *et al.*, 2010). Since SP is the subject of the present thesis, this will be discussed in more detail in the next section.

2. SUCROSE PHOSPHORYLASE

2.1 Mechanism and classification

Sucrose phosphorylase (SP) catalyzes the reversible phosphorolysis of sucrose into α-D-glucose-1-phosphate and fructose, following a double displacement mechanism (Mirza *et al.*, 2006). Just as with retaining glycosidases, two carboxylic amino acids are involved in the mechanism, *i.e.* a catalytic acid and a catalytic nucleophile (Figure 1.6). Although a catalytic base is not required in the second step, the deprotonated acid is unavoidably present at the corresponding position. Its activity can result in the activation of a nucleophilic water molecule, explaining why sucrose phosphorylase also catalyzes a minor hydrolytic side reaction (Wiesbauer *et al.*, 2009).

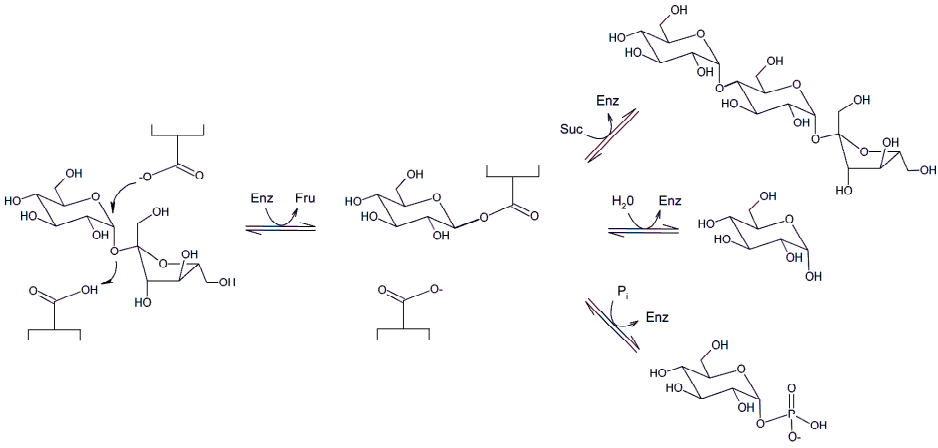

Figure 1.6 Reactions catalyzed with sucrose by GH-13 enzymes. From the covalent-intermediate, a glucosyl group can be transferred to amylose, water or inorganic phosphate.

All SP enzymes are classified in family GH-13, one of the largest families in the CAZY database (Stam *et al.*, 2006). It mainly contains α-amylases but also a number of enzymes that catalyze transglucosylation reactions from amylose (*e.g.* trehalose synthase), as well as a group of enzymes that use sucrose as substrate, *i.e.* sucrose hydrolase, sucrose phosphorylase and amylosucrase. This means that based on the same structural scaffold and retaining mechanism, a

variety of reactions with sucrose can be performed (Figure 1.6). Furthermore, all three enzymes have been shown to display low activity towards non-carbohydrate acceptors as well. The determinants of the acceptor specificity in GH-13 are not well understood but their study should eventually allow to create tailor-made biocatalysts for the glycosylation of specific target molecules.

2.2 Properties and applications

Sucrose phosphorylase is an intracellular enzyme that was discovered in the 1940s independently in *Leuconostoc mesenteroides* by Kagan and co-workers (Kagan *et al.*, 1942) and in *Pseudomonas saccharophila* by Doudoroff and collaborators (Doudoroff, 1943; Doudoroff *et al.*, 1943). Since then, it has been found in many micro-organisms, especially *Bifidobacterium* species, *Pseudomonas* species and *Leuconostoc mesenteroides*. Most SP enzymes consist of around 500 amino acids and were found to occur as either a functional monomer or dimer (Table 1.4).

Table 1.4 An overview of known sucrose phosphorylases

Micro-organism	M_w (kDa)	T_{opt} (°C)	pH_{opt}	Functional state	Reference
Leuconostoc mesenteroides	55.8	37[a], 42	6.5 - 7	monomeric	(Koga *et al.*, 1991; Goedl *et al.*, 2007)
Bifidobacterium adolescentis	56.2	48	6 - 6.5	dimeric	(van den Broek *et al.*, 2004)
Bifidobacterium lactis	55.7	-	-		(Trindade *et al.*, 2003)
Bifidobacterium longum	56	-	-		(Kim *et al.*, 2003; Shin *et al.*, 2008)
Streptococcus mutans	-	45	6 – 6.5	monomeric	(Fujii *et al.*, 2006; Sugimoto *et al.*, 2007)
Pseudomonas saccharophila	80-100	-	7.0[b]	dimeric	(Silverstein *et al.*, 1967)
Pseudomonas putrefaciens	-	-	6.6 - 6.8[b]		(Weimberg & Doudoroff, 1954)
Pseudobutyrivibrio ruminis	52	45	6		(Kasperowicz *et al.*, 2009)

[a] Synthesis direction; [b] Phosphorolysis direction.

The acceptor specificity of SP from *L. mesenteroides* and *B. longum* was shown to be remarkably broad, especially in comparison with other phosphorolytic enzymes. Not only various sugars act as good acceptors, but also non-carbohydrate acceptors like aliphatic, aromatic and sugar alcohols, hydroxybenzene, furanones, carboxylic compounds (fatty acids, dicarboxylic acids, hydroxy acids, aromatic acids), catechines and phenolics (Mieyal *et al.*, 1972; Kitao *et al.*, 1993; Kitao & Sekine, 1994b; Kitao & Sekine, 1994a; Kitao *et al.*, 1995; Kitao *et al.*, 2000; Kwon *et al.*, 2007). Even a carboxyl group (e.g. acetic and caffeic acid) can be used as point of attachment, resulting in an ester instead of an ether bond (Sugimoto *et al.*, 2007; Nomura *et al.*, 2008; Shin *et al.*, 2009). However, disaccharides or higher oligosaccharides have not yet been reported as acceptors for SP (van den Broek *et al.*, 2004). In comparison with the acceptor specificity, the donor specificity of SP is relatively narrow. Only sucrose, α-D-glucose-1-phosphate and α-glucosyl fluoride have been reported as donor substrates (Goedl *et al.*, 2007).

The broad acceptor specificity of SP has already been exploited for various synthetic applications. Examples include the production of anti-cancer furanone glucosides with *L. mesenteroides* SP (Kitao *et al.*, 2000) and the glucosylation of ascorbic acid with *B. longum* SP (Kwon *et al.*, 2007). In the latter case, the glycosylated product displays improved stability but retains its anti-oxidative activity after *in vivo* hydrolysis by α-glucosidase. Another example of the glucosylation of an anti-oxidant is the synthesis of glucosides of caffeic acid with *B. longum* SP (Shin *et al.*, 2009). Recently, an extremely efficient process for the production of 2-*O*-(α-D-glucopyranosyl)-*sn*-glycerol has been described (Goedl *et al.*, 2008). Under the right conditions, the competing hydrolytic reaction could be completely suppressed, resulting in near quantitative yields. The product is commercially available under the trade name Glycoin® and can be used as moisturizing agent in cosmetic formulations but also as a low-calorie sweetener for the prevention of tooth decay.

Because SP follows a double displacement mechanism, it can be applied as a transglycosidase without the participation of (glycosyl) phosphate. In that case, sucrose is used as donor substrate, from which a glucosyl group is transferred to an acceptor molecule. Sucrose is a very reactive disaccharide that allows yield to be obtained comparable with those of the nucleotide-activated donors of glycosyl transferases, making SP unique among glycoside phosphorylases (Monsan & Ouarne, 2010). Nevertheless, its phosphorolytic reaction has also been exploited for synthetic applications. For example, the production of α-D-glucose-1-phosphate (αG1P) from sucrose and inorganic phosphate has been carried out successfully in a continuous packed-bed reactor with the immobilized *L. mesenteroides* SP as biocatalyst (Goedl *et al.*, 2007). Furthermore, SP is often used for the *in* situ generation of αG1P to be used as donor for a second GP enzyme, in an approach known as phosphorylase coupling (Goedl *et al.*, 2010).

2.3 Crystal structure

Structural information is only available for the SP from *B. adolescentis* (Sprogoe *et al.*, 2004). This enzyme is known to form a functional dimer in solution (van den Broek *et al.*, 2004), which was also observed in its crystal structure (PDB entry 1r7a). Each monomer consists of 4 separate domains, with the catalytic domain (referred to as domain A) adopting the canonical $(\beta/\alpha)_8$-barrel fold of family GH-13 (Figure 1.7).

Figure 1.7 The $(\beta/\alpha)_8$-barrel of the catalytic domain A of *B. adolescentis* SP

Apart from the catalytic domain, SP from *B. adolescentis* is composed of three other domains, known as B, B' and C (Figure 1.8). Domain B contains two short anti-parallel β-sheets and two short α-helices, whereas domain B' is mainly a coil region, but also contains one long and one short α-helix. The first 56 residues of the C-terminal domain form a single five-stranded anti-parallel β-sheet with a topology described as 1,1,1,1 in algebraic notation and this is unique among family GH-13 enzymes.

The majority of the subunit interactions are confined to the B domains, but interactions between the loop 8 regions of the A domains also observed. This results in a large cavity in between the monomers that forms the entrance to the two active sites (Figure 1.8). Both catalytic centers contain two conserved amino acids that function as catalytic residues, *i.e.* the catalytic nucleophile Asp192 and the catalytic acid Glu232. These catalytic residues can be found at the tips of β-sheet 4 and 5 in the $(\beta/\alpha)_8$-barrel of SP.

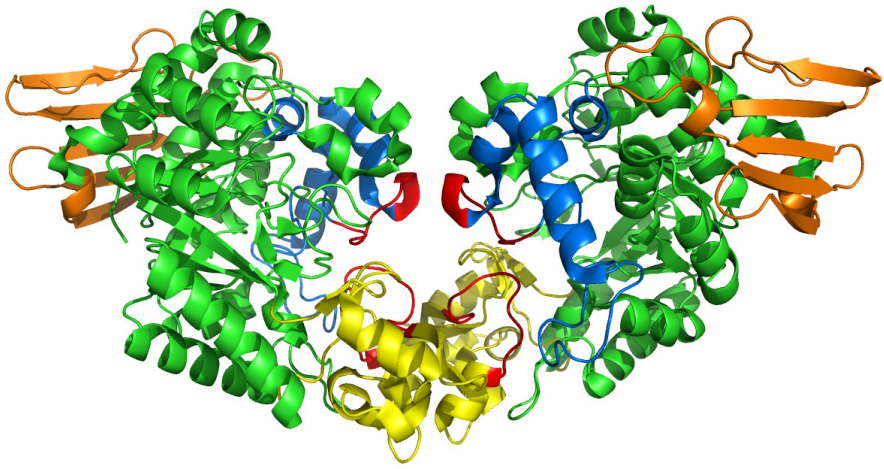

Figure 1.8 The homodimeric structure of SP from *B. adolescentis* (PDB 1r7a). Domains A, B, B' and C are colored in green yellow, blue and orange, respectively. Loops A and B are shown in red.

Mirza and co-workers brought further insight in the catalytic mechanism of SP by determining the structure of various enzyme-ligand complexes (Mirza *et al.*, 2006). When sucrose was co-crystallized, the intact substrate could not be detected because it was enzymatically processed, even in the absence of phosphate. In one monomer, the covalent glycosyl intermediate was observed, while free glucose was present in the other (PDB 2gdv). Therefore, an inactive enzyme variant was created by substituting the catalytic acid (E232N), which resulted in a structure for the binary Michaelis complex (PDB 2gdu). These snapshots along the reaction coordinate provided detailed information on the mechanism of SP and in particular on the importance of specific loop movements.

In the covalent glycosyl-enzyme intermediate, the carbohydrate ring was found to adopt a $^{1,4}B$ boat conformation (Figure 1.9). Such distortion has been observed in many glycosidase complexes of and is believed to be required for maximal electronic and steric reactivity of a β-glycosidic bond (Vocadlo & Davies, 2008). More importantly, in the product complex, loops A and B have drastically changed their orientation, bringing Arg135 and Tyr344 into the acceptor subsite. These residues are then optimally positioned for the binding of a phosphate ion, and probably help the latter to outcompete water as nucleophile.

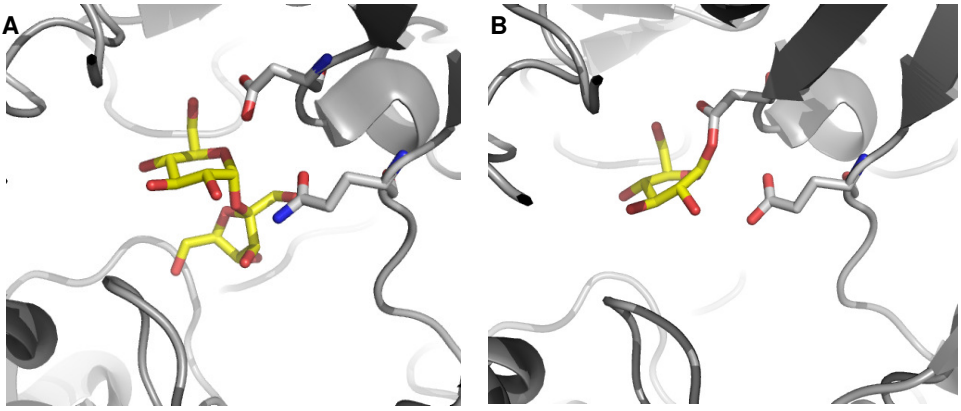

Figure 1.9 Active site structure of SP from *B. adolescentis*. A. SP(E232Q)-sucrose complex (PDB 2gdu) B. SP-glucosyl intermediate complex (PDB 2gdv).

Interestingly, the observed asymmetry between both monomers (one covalently bound to glucose, the other containing free glucose) may reflect the way that phosphate ions are brought to the active site. It implies that SP has only one surface phosphate-binding site from which phosphate ions are transported to the active site by Arg135. Thus, *Ba*SP might work in such a way that the two monomers are out of phase and require phosphate at different times; whereas one monomer phosphorylates the covalent intermediate and releases glucose 1-phosphate, the other monomer binds sucrose and creates the covalent intermediate.

3. THERMOSTABILITY

3.1 Introduction

The increasing interest in applying enzymes in industrial processes has spurred the search for biocatalysts with new or improved properties (Kirk *et al.*, 2002; Turner, 2003). The major advantages of the use of enzymes for chemical transformations are their high chemo-, regio- and stereospecificity, as well as their environmentally friendly properties. Unfortunately, natural enzymes are often not optimally suited for industrial applications, which can be hampered by their lack of long-term stability under process conditions (e.g. presence of organic solvents, extremes of pH or high temperatures). Although it often is beneficial to adapt industrial processes to the mild and environmentally benign conditions favored by the enzyme, the use of more extreme conditions is sometimes required. Regardless of process conditions, the stability of the biocatalyst typically is a crucial determinant of economic success (Eijsink *et al.*, 2005). Hence, the development of enzymes with higher stability will increase the adoption of biocatalytic syntheses in industrial production.

Stability of an enzyme is affected by many factors, such as temperature, pH, oxidative stress, binding of metal ions or co-factors and the presence of organic solvent or surfactants. The effect of surfactants is extremely important from an industrial point of view since the detergent area is the largest application area of industrial enzymes. Stability in the presence of organic solvents, in turn, is essential when applying enzymes for the production of fine chemicals (Luetz *et al.*, 2008). Of all potentially deactivating factors, temperature has been studied most extensively and its effect will be described in this section. Additionally, there is some evidence that proteins that are stable to one type of denaturing condition are also stable towards other types (Cowan, 1997; van den Burg *et al.*, 1998; Wang *et al.*, 2000b; D'Amico *et al.*, 2003). However, this correlation is not absolute, especially when it comes to denaturation processes which do not or to a minor extent depend on folding stability.

Thermostability is one of the most important properties of industrial enzymes, especially for carbohydrate conversions. The main advantage of working at high process temperatures is reduction of microbial contamination but it may also be beneficial for substrate and product solubility, viscosity and process speed (Haki & Rakshit, 2003). High temperatures also result in a decreased level of secondary reactions taking place (Vogt *et al.*, 1997). In addition, thermostable enzymes are often easier to purify as contaminating proteins can simply be removed by incubation at high temperature (Becker *et al.*, 1997). Unfortunately, at elevated temperature

many enzymes become (partly) unfolded and/or inactivated, meaning that they are no longer able to perform the desired tasks. This can be caused by incompatibility of the optimum temperature for activity or relate to the intrinsic stability of the enzymes (Danson *et al.*, 1996; Daniel *et al.*, 2001; Peterson *et al.*, 2004).

3.2 Kinetic versus thermodynamic stability

Over time, the term 'protein stability' has come to have different meanings, often leading to confusion and ambiguity in comparing results from the literature. It encompasses both thermodynamic and kinetic stabilities (Vieille & Zeikus, 2001; Eijsink *et al.*, 2005). The former relates to the fraction of proteins that is present in the folded state at a specific temperature. These folded proteins are in equilibrium with the unfolded state, as determined by their difference in Gibbs free energy (ΔG_{stab}). Kinetic stability, in contrast, relates to the time it takes a protein to denature irreversibly, mainly due to aggregation and proteolytic degradation. In that case, the partially or fully unfolded protein undergoes a permanent change so that its structure cannot be restored.

The study of the tendency of a protein to reversibly unfold is of great importance for understanding the principles of protein structure and stability (Matthews, 1995). Unfolding is a measure of thermodynamic stability and is characterized by the enzyme's melting temperature (T_m), which is the temperature at which 50 % of the protein is unfolded. Methods of measuring thermodynamic stability include differential scanning calorimetry (DSC), circular dichroism (CD), tryptophan fluorescence or changes in tyrosine absorbance. We should, however, keep in mind that many proteins do not unfold reversibly and if they do so in the laboratory, they do not necessarily behave likewise in the more complex environment of an enzyme reactor. Therefore, for most industrial enzymes, kinetic stability is more relevant than thermodynamic stability.

The most commonly reported measure of a protein's kinetic stability is its half-life ($t_{1/2}$) of denaturation, or the time it takes for the activity of the enzyme to be reduced by half at defined temperatures (Polizzi *et al.*, 2007). Other measures of kinetic stability include the optimum operating temperature (T_{opt}) and T_{50}, the temperature at which the activity of the enzyme is reduced by half after incubation for a specific amount of time. Measurements of kinetic stability usually rely on an assay for the enzymatic activity of interest. Although unfolding and deactivation can sometimes be related (often the former is a contributing factor to the latter), they clearly refer to different processes and are quantified differently.

There are different strategies to improve the protein stability, both thermodynamic and long term (Vieille & Zeikus, 2001; Polizzi *et al.*, 2007). Chemical stabilization methods using additives, immobilization or a combination of both have all proven to be successful (Bornscheuer, 2003; Fernandez *et al.*, 2005; Grazu *et al.*, 2005; Sangeetha & Abraham, 2006; Villalonga *et al.*, 2006). Different immobilization techniques will be discussed in the next section of this chapter. Biological approaches to obtain more stable will be discussed here in more detail.

3.3 Improve the protein stability

The advent of recombinant DNA technology has led to different strategies for the identification and creation of enzyme variants with improved stability. There are basically two routes to obtain such variants: the screening of (meta)genomes isolated from extreme environments for the presence of new enzymes (van den Burg, 2003), or protein engineering to increase the stability of existing biocatalysts (Turner, 2003; Eijsink *et al.*, 2004).

3.3.1 Screening in nature

The overwhelming biochemical diversity present in nature makes the search for micro-organisms with improved properties worthwhile. In order to find a specific biocatalyst that is adapted to the required process conditions, one should sample at a site where similar conditions apply. Such sampling sites have a lower biodiversity but a higher selection pressure. When searching for thermostable enzymes, important sites are for example hot springs and volcanic environments. However, one of the major problems in classical screening is that only about 1 % of the micro-organisms that occur in nature can be cultured under laboratory conditions, which drastically limits access to the existing biodiversity. This situation has spurred the development of metagenomics, a culture-independent approach to isolate, clone and characterize microbial genomes and the enormous genetic pool of 10^{33} putative genes in the environment (Beloqui *et al.*, 2008). Metagenomics can accelerate the discovery of novel biocatalysts by enabling access to genes from uncultivable extremophilic micro-organisms (Figure 1.10). The organisms and their enzymes have been adapted over millions of years to extreme conditions such as high temperatures. Therefore, their enzymes hold great potential for industrial applications.

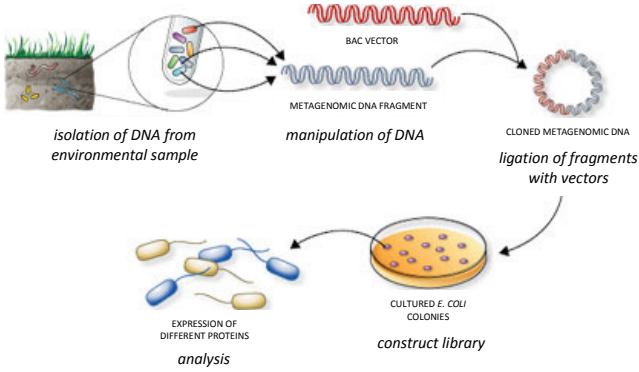

Figure 1.10 The standard steps of a metagenomics experiment. Environmental DNA is fragmented and cloned into a suitable vector for recombinant expression by a cultivable micro-organism.

The metagenomics approach was successfully applied to isolate a thermostable esterase from environmental samples collected from Indonesian hot springs and mud holes (Rhee *et al.*, 2005). The T_{opt} of the esterase was 95 °C and there was almost no loss in activity after 2 hours incubation at 80 °C. Similarly, He and colleagues discovered a thermostable Fe-superoxide dismutase from a metagenomics library from a hot spring (He *et al.*, 2007). The enzyme was highly stable at 80 °C and retained 50 % activity after heat treatment at 95 °C for 2 hours.

3.3.2 Protein engineering strategies

Protein engineering approaches to generate more stable biocatalysts can be divided into three main categories: rational-based mutagenesis (Eijsink *et al.*, 2004), directed evolution (Eijsink *et al.*, 2005) and semi-rational design (Chica *et al.*, 2005). Depending on the availability of the 3D structure and knowledge about the mechanism of deactivation, one or several of these strategies can be selected to improve an enzyme's stability.

3.3.2.1 Rational design

Rational design incorporates the 3D structure of the enzyme along with mechanistic knowledge to design specific amino acid substitution that will hopefully lead to the desired outcome. Applying site-directed mutagenesis to increase the stability of a protein requires knowledge of the mechanism of deactivation so that substitutions can be aimed at specific problem areas. For example, the thermostability of the tetrameric lactate oxidase from *Aerococcus viridians* was increased by mutations designed to strengthen subunit interactions (Kaneko *et al.*, 2005). A homology model of the enzyme was used to identify a pair of interfacial glutamate residues with a potential electrostatic repulsion that could destabilize the quaternary structure of the protein.

Substituting one of these residues to a glutamine led to an almost two-fold increase of half-life at 65 °C.

Although there are many examples of enzymes that have been stabilized by introduction of only one or two mutations (Mansfeld *et al.*, 1997; Williams *et al.*, 1999; Gerk *et al.*, 2000; Sandgren *et al.*, 2003; Bjork *et al.*, 2004) and despite many successful efforts to understand the structural basis of protein stability, there is still no universal strategy to stabilize ‘‘any’’ protein by a limited number of rationally designed mutations (Eijsink *et al.*, 2004). Well-known and reasonably successful types of rational design include rigidifying mutations (e.g. Xxx → Pro or Gly → Xxx or the introduction of disulphides), working primarily through their effect on the entropy of the unfolded state, improvement of molecular packing, modification of surface charge networks or reinforcement of a higher oligomerisation state (Matthews, 1995; van den Burg & Eijsink, 2002; O'Fagain, 2003).

3.3.2.2 Directed evolution

In contrast to rational design, directed evolution does not require knowledge about the mechanism of deactivation and does not rely on the crystal structure of the enzyme. The basic principle is an iterative process of random mutagenesis and screening of huge libraries of variants (typically ~10^5) to find an enzyme with improved properties (Figure 1.11). In that respect, directed evolution mimics Darwinian evolution in a test tube, in which the best enzyme variant is selected by means of ‘survival of the fittest’. In the laboratory however, the process is applied to one gene of interest and not to the complete genome of an organism. Several methods are available to introduce random mutations in DNA. These are mostly based on molecular biology techniques, although classical chemical mutagens have also been used.

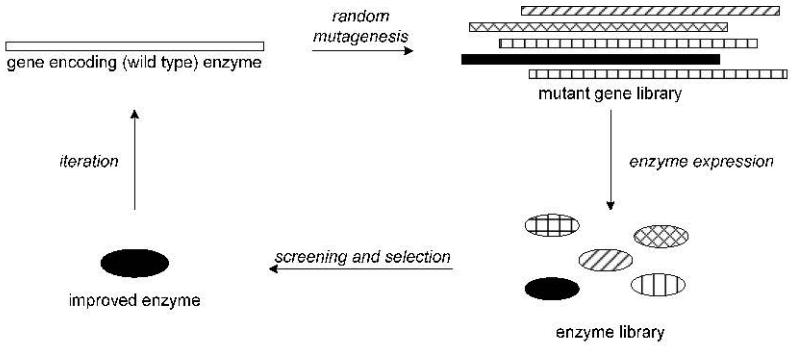

Figure 1.11 Schematic representation of directed evolution (De Groeve, 2009). A library of mutant genes is generated and transformed into a suitable expression host for selection or high-throughput screening of the corresponding enzyme variants.

Based on molecular biology techniques, two different strategies can be used for the generation of enzyme variants: either random mutagenesis ('asexual' evolution) or DNA shuffling ('sexual' evolution). In the former strategy, random mutations are introduced in one gene that codes for a wild-type protein. Sexual evolution, in contrast, starts from a pool of homologous parent genes from which new combinations are randomly created.

The most common technique for 'asexual' evolution is error-prone PCR (epPCR) (Pritchard *et al.*, 2005). This technique exploits the fact that DNA polymerases such as Taq polymerase occasionally make errors during the DNA replication process and so introduce random mutations in DNA during PCR. Although the natural error rate is relatively low, it can be increased by adding $MnCl_2$ to the reaction mixture or by using unbalanced dNTP concentrations (Cadwell & Joyce, 1992; Neylon, 2004). This *in vitro* method is very easy to perform but suffers from a pronounced mutational bias of the polymerases, which favor replacement of As and Ts. Another limitation of epPCR is that some mutations are almost impossible to access, especially at low mutagenesis rates. Indeed, mutating e.g. Phe into Glu would require 3 consecutive DNA mutations, something that is unlikely to happen during epPCR. Despite its restrictions, epPCR was shown to be a very successful strategy for the enhancement of thermostability. Fujii and co-workers increased the thermal stability of sucrose phosphorylase from *S. mutans* using epPCR. Random mutagenesis revealed that eight single amino acid substitutions contributed to the thermostability of SP. The SP variant comprising all eight mutations retained more than 60 % of its initial activity after 20 min incubation at 60 °C whereas the wild-type SP lost all its activity (Fujii *et al.*, 2006).

In 'sexual' evolution, different enzyme variants or homologous enzymes are recombined using molecular tools. The most popular technique for recombining diverse sequences is DNA shuffling (Stemmer, 1994a; Stemmer, 1994b; Neylon, 2004). It is based on a partial DNase digestion of homologous genes followed by a recombination of fragments by PCR. The parental genes can originate from an epPCR library, from related natural sequences or from enzyme variants generated by rational design. This procedure allows a more complete coverage of the sequence space but is more difficult to perform than epPCR. Numerous cases have been reported where DNA shuffling, sometimes preceded by epPCR, was used to increase the thermostability of an enzyme. Xia and Wang increased the thermal stability of *S. lividans* xylanase B using three rounds of evolution. The variant remained active at 70 °C for 6 h, while the wild-type enzyme lost 50 % of its activity after only 3 min (Xia & Wang, 2009). Also the thermostability of a β-agarase was successfully increased by directed evolution. The mutant, obtained through two

rounds of epPCR and a single round of DNA shuffling, showed an increase in T_m of 4.6 °C and an 18.4-fold higher $t_{1/2}$ value at 40 °C (Shi *et al.*, 2008).

3.3.2.3 Semi-rational design

A very interesting and powerful approach to optimize an enzyme's function consists of a combination of rational design and directed evolution. Indeed, so-called semi-rational design uses the available knowledge to limit library size while still allowing for the identification of unpredictable substitutions that lead to large effects. More specifically, a specific amino acid (hot spot) is targeted for saturation mutagenesis, meaning that all 20 amino acids are tested at this position. Site-saturation mutagenesis (SSM) can be achieved in an amplification reaction using mutagenic primers with degenerate codons. The QuikChange mutagenesis kit from Stratagene (USA) is one of the most popular to create saturation mutagenesis libraries. The most frequently used degenerate codons are NNS and NNK, each generating 32 possible codons that encode all 20 amino acids and only 1 stop codon (appendices I-III). Recently, it was shown that smaller libraries whereby the number of generated codons equals the number of possible amino acids (e.g. NDT codon), can be of better quality and require less screening effort to find improved mutants (Reetz *et al.*, 2008; Reetz & Wu, 2008).

The 'B-fit' method developed by Reetz and coworkers is one such example of semi-rational design for increased thermostability (Reetz *et al.*, 2006). It specifically targets positions with high flexibility for saturation mutagenesis. These can be identified on the basis of B-factors, *i.e.* atomic displacement parameters obtained from x-ray data that reflect smearing of atomic electron densities with respect to their equilibrium positions as a result of thermal motion and positional disorder. Consequently, only the amino acids with the highest B-factors are subjected to randomization, resulting in small focused libraries of enzymes variants. Libraries can be generated from a single position or from a group of neighboring positions to allow for synergistic effects. After the screening of one library, the best hit is subsequently used as a template for a second round of saturation mutagenesis and the process is repeated iteratively until the desired level of improvement is achieved. Impressive results have been obtained for a *Bacillus subtilis* lipase, of which the T_m was increased from 48 °C to 93 °C by iterative site-saturation mutagenesis of 7 amino acid residues (Figure 1.12).

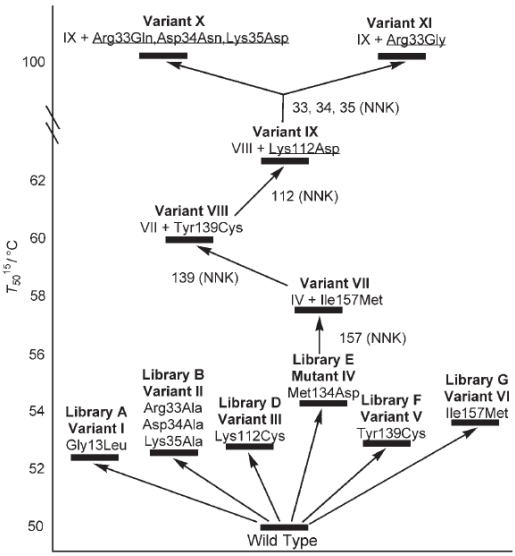

Figure 1.12 Results of the B-FIT strategy applied on *Bacillus subtilis* lipase (Reetz *et al.*, 2006). By iterative site-saturation mutagenesis of 7 amino acid positions with high B-factors, the T_m was increased with nearly 50 °C.

3.3.3 Screening for thermostability

To screen enzymes for high thermostability, measuring activity at elevated temperatures can be an effective method. Indeed, enzymes that withstand higher temperatures usually also have higher temperature optima for activity (van den Burg *et al.*, 1999; Zhao & Arnold, 1999). However, exceptions have been reported (Arnott *et al.*, 2000; Bjork *et al.*, 2003) and it has been shown that temperature optima do not necessarily give an indication of what will be measured in assays of conformational stability (Daniel *et al.*, 2001; Peterson *et al.*, 2004). Furthermore, high-throughput screening is preferably performed in microplate readers, which could result in a considerable amount of evaporation at high temperatures.

A more convenient way of measuring an enzyme's thermostability is to determine its residual activity at room temperature after incubation at elevated temperatures (Cirino & Georgescu, 2003; Robertson & Steer, 2004). To allow the processing of large libraries of enzyme variants, a simple, rapid and accurate detection system has to be available (Bommarius *et al.*, 2006). Many screening systems are based on colorimetric tests that can be followed spectrophotometrically. However, chromogenic substrate analogues should be avoided because these not always reflect

the enzyme's activity on the true substrate of interest (Schmidt & Bornscheuer, 2005). This is famously referred to as the first law of directed evolution: 'You get what you screen for' (Schmidt-Dannert & Arnold, 1999).

In some studies, the improvement in thermostability was measured using DSC rather than by the methods described above. Increases in reaction T_{opt} are often higher (most likely due to substrate stabilization effects) than shifts in T_m as measured by techniques such as DSC that directly measure thermostability. Stabilized protein variants can also be selected by methods based on phage display. One such approach is 'Proside' (protein stability increased by directed evolution), where the protein of interest is spliced into the multidomain capsid protein important for phage infectivity (Sieber *et al.*, 1998). Infectivity is lost when the domains are disconnected by proteolytic cleavage of unstable protein inserts. The method is best suited to small, monomeric proteins, as it requires that the insertion does not abolish the assembly or infectivity of the phage. Although the stability of the phage limits the stabilization of the protein of interest achieved with the Proside method, phages with improved thermotolerance (+20 °C) have been engineered (Martin & Schmid, 2003).

The use of selection methods for increased stability is still very rare. However, thermophilic bacteria can be employed as host to perform the selection at higher temperatures. Tamakoshi and co-workers used the thermophile *Thermus thermophilus* directly as a selection system. A gene from *Saccharomyces cervisiae* encoding one of the enzymes in leucine biosynthesis was cloned into a leucine negative strain of *T. thermophilus*. After diversity generation, transformants that could grow without leucine were selected at increasingly higher temperatures. Mutations accumulated serially and the final five-fold mutant showed a considerable increase in thermal stability (Tamakoshi *et al.*, 2001).

4. IMMOBILIZATION

A number of enzymes are too unstable or too expensive to allow their commercial exploitation. These problems can at least partially be solved by binding them to or within a matrix, i.e. by enzyme immobilization (Hanefeld *et al.*, 2009). The main purpose of this process is to make the biocatalyst insoluble and behave like a heterogeneous catalyst, which can be used in repeated batches or in a continuous-flow reactor. Indeed, insoluble enzymes can be easily separated from the reaction medium and recycled (by *e.g.* filtration or centrifugation) or can be packed in column through which a substrate solution is continuously pumped. Both strategies result in a prolonged use of the enzyme, which can improve the economics of the process if the enzyme is expensive. Additionally, immobilization prevents contamination of the product with enzyme, which can be crucial for applications in the pharmaceutical or food sector (Buchholz *et al.*, 2005).

Another important advantage of immobilization is the improvement in enzyme stability as their three-dimensional structure is 'fixed' in space and denaturation can be slowed down. In that way, immobilization improves enzyme performance under optimal process reaction conditions (*e.g.* acidity, alkalinity, organic solvents and elevated temperatures), a requirement that has often retarded enzyme application in industrial chemical synthesis (Bommarius & Riebel, 2004). Some processes employing insoluble enzymes suspended in hydrophobic organic media require immobilization to optimize enzyme dispersion to improve accessibility for the substrates, as well as to avoid the aggregation of the hydrophilic protein particles. Finally, improvements in (enantio)selectivity has also been observed upon immobilization (Palomo, 2008; Wang *et al.*, 2008; Cabrera *et al.*, 2009).

The importance of immobilization is illustrated by the fact that about half of the biocatalytic processes at the industrial scale make use of immobilized enzymes. The two most important examples are glucose isomerase for the production of high fructose corn syrup (HFCS) and penicillin amidase for the synthesis of semi-synthetic antibiotics. The latter enzyme is extremely unstable when removed from its cellular environment and can, therefore, only be applied in immobilized form, which also allows its recycling for hundreds of batch reactions (Kallenberg *et al.*, 2005). The former enzyme, in contrast, is relatively stable but is needed in large quantities to compensate for its high K_m for glucose. The associated costs can be substantially reduced by applying the immobilized enzyme in a continuous process avoiding contamination of the food product with the biocatalyst (Bhosale *et al.*, 1996).

Possible drawbacks of enzyme immobilization are the cost of the carriers, the 'dilution of activity' within the matrix and slow diffusion of substrate and product to and from the enzyme's active site. The kinetic constants (k_{cat}/K_m) of an immobilized enzyme can therefore differ from the ones of the enzyme in solution and should always be verified experimentally. During immobilization, part of the activity (up to 90 %) might be lost. The loss of activity depends on the immobilization method. Less activity is lost in 'gentle' systems such as gel entrapment, whereas more activity will be lost in aggressive immobilization methods, *e.g.* covalent attachment. On the other hand, the stabilizing effects will be more pronounced if stronger interactions between the enzyme and the carrier exist. Which effect will dominate the final outcome is not easy to predict and usually has to be determined experimentally.

Several strategies exist for the immobilization of biocatalysts and these will discussed in the next paragraphs. The final outcome of each method is actually a balance between the loss of activity due to immobilisation, the gain of activity resulting from stabilisation effects, leakage and possible diffusing limitation. Furthermore, it is also possible to recycle an enzyme in soluble form by using a semi-permeable membrane. The most important example of the latter strategy is a membrane reactor, but semi-permeable microcapsules (encapsulation) have also been described. An overview of the commonly used techniques is presented in Figure 1.13.

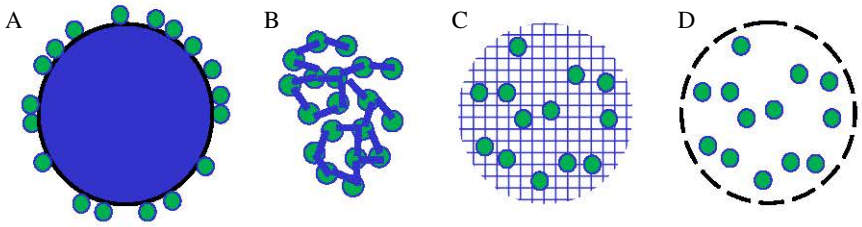

Figure 1.13 Different strategies for enzyme immobilization. A. binding to an insoluble carrier, B. crosslinking of enzyme molecules, C. entrapment in an insoluble matrix, D. encapsulation in semi-permeable microcapsules.

4.1 Immobilization techniques

4.1.1 Carrier-bound immobilized enzymes

The standard method for enzyme immobilization is binding to insoluble porous carriers (Hartmeier, 1988). An unavoidable consequence of this procedure is that the activity will be diluted, as carriers can account for 90 - 99 % of the mass of the resulting biocatalyst (Lalonde & Margolin, 2002; Sheldon, 2007). The properties of supported enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two

provides an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties.

Carriers most often come in the form of mesoporous beads. The pores allow the enzymes to access the inner surface of the beads, which drastically increases the amount of enzyme that can be immobilised. The internal surface typically is about 100 m^2 g^{-1} and the pore diameter should be about 100 nm. The beads themselves have a size of about 0.5 mm; larger particles result in a lower activity due to increased mass-transfer limitations while smaller particles increase the resistance in a plug-flow reactor. In any case, the particle size should be as uniform as possible to ensure optimal flow in the reactor.
Enzymes can be linked to the carriers either by adsorption or by covalent bonds. The former method is the simplest and is inexpensive. It is capable of high enzyme loadings (about one gram per gram of matrix) and does not chemically modify the enzyme. The driving force is a combination of hydrophobic interactions and the formation of ionic bonds (salt links). Although the physical links between the enzyme and the support can be very strong, leakage of the enzyme is often a problem and should be minimised as much as possible by selecting the appropriate reaction conditions (especially the pH). Examples of suitable adsorbents are clays, glasses, polymeric aromatic resins and ion-exchange matrices. The latter type is the most expensive but can be easily regenerated when their bound enzyme has come to the end of its active life and is therefore economically attractive. Commercially available ion-exchangers are composed of either polystyrene (*e.g.* Dowex), polyacrylesters (*e.g.* Amberlite) or polysaccharides (*e.g.* Sephadex) modified with carboxyl groups or tertiary amines.

Table 1.5 Overview of carriers used for covalent immobilization and their mode of activation

Carrier type	Examples	Functionality	Activation
Inorganic (SiO_2-based)	porous glass porous silica (*Deloxan*) aluminosilica	-OH	APTS + GA
Biological (polysaccharides)	agarose (*Sepharose*) dextran (*Sephadex*) cellulose	-OH	CNBr
Organic (synthetic)	acrylic copolymer *(Eupergit)* aromatic copolymer *(Sepabeads)* polyamide *(Nylon)*	Epoxide Various $-NH_2$	(reactive) (reactive) GA

Immobilization through covalent coupling results in much stronger interactions with almost no leakage, but the enzyme loading capacity is lower (about 0.1 gram per gram of matrix). Lysine

residues are the most useful groups for covalent binding of enzymes, due to their widespread surface exposure and the high reactivity of their amino-group. Alternatively, the carboxyl groups of Asp/Glu or the hydroxyl groups of Ser/Thr/Tyr can be employed. Carriers that can be used for covalent immobilization typically consist of insoluble polymers, which are either inorganic (silica-based), biological (polysaccharide) or organic (synthetic) (Table 1.5). The first type has long been the most popular, due to its low cost and ease of activation. Indeed, the hydroxyl groups of silica can be conveniently derivatised with aminopropyl triethoxysilane (APTS) to introduce amino-groups that can be coupled to lysine residues with glutaraldehyde. The most commonly used method for immobilizing enzymes on a laboratory scale involves Sepharose, activated by cyanogen bromide (Figure 1.14a). However, the high toxicity of cyanogen bromide has fuelled the search for safer alternatives.

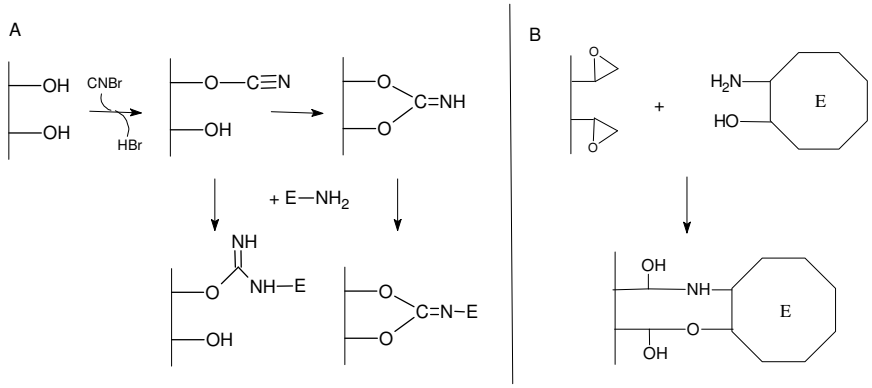

Figure 1.14 Covalent attachment to CNBr-activated sepharose (A) and Eupergit (B).

Epoxy-activated supports such as Eupergit (Rohm/Degussa) and Sepabeads (Mitsubishi/Resindion) are almost-ideal matrixes to perform a covalent immobilization of proteins both in academia and the industry. These carriers are made up of synthetic polymers that are very stable under a wide range of conditions and do not suffer from abrasion when stirred. They are commercially available in an activated form, and do not need further derivatisation before use. It has been described that soluble proteins are scarcely reactive with epoxy-groups at neutral pH values. This low reactivity causes the immobilization of enzymes on these supports to be produced via a two-step mechanism: first, rapid and mild physical adsorption of the protein on the support; secondly, a covalent reaction between the adsorbed protein and neighbouring epoxy-groups occurs (Mateo *et al.*, 2002; Mateo *et al.*, 2007) (Figure 1.15). Covalent linkages are not only formed with lysine but also with other amino acids (Figure 1.14b). Due to the high density

of reactive groups present on these carriers, an enzyme can be bound by more than one covalent linkage per molecule. Such 'multi-point' covalent attachment typically results in an exceptional operational stability of the immobilized enzyme. Epoxy-groups that did not react with the enzyme can be blocked with glycine or mercaptoethanol in order to prevent uncontrolled reaction between the enzyme and support (Mateo *et al.*, 2003; Hildebrand & Lutz, 2006).

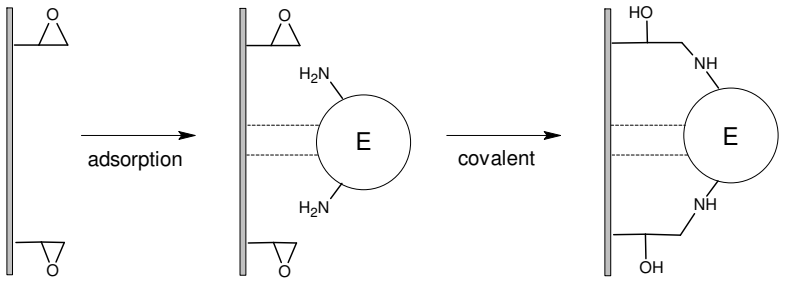

Figure 1.15 Immobilization of proteins on epoxy-activated supports. The protein rapidly binds to the support by physical adsorption, after which multiple covalent linkages are slowly formed with the epoxy-functionalities.

Conventional (first generation) epoxy-supports exhibit several limitations. Proteins should remain active and soluble in the high salt concentrations that are required for adsorption to the hydrophobic support. Furthermore, proteins are immobilized via their most hydrophobic parts, which usually contain a low amount of nucleophiles. This gives rise to a low multipoint covalent attachment between the enzyme and the support, resulting in a low stabilization effect (Mateo *et al.*, 2007). Moreover, very hydrophilic proteins cannot be immobilized at all (*e.g.* highly glycosylated proteins). In fact, it has been described that only approximately 70 % of the proteins present in crude enzyme preparations could be immobilized on these type of supports (Mateo *et al.*, 2000). These drawbacks lead to the development of a new generation of epoxy-activated supports.

This second generation epoxy-activated supports contains two types of functional groups: groups able to promote the physical adsorption of proteins (e.g. ethylenediamine) and groups able to covalently immobilize the enzyme (*e.g.* epoxy-groups) (Figure 1.16b) (Mateo *et al.*, 2003; Mateo *et al.*, 2007). As a consequence, fewer groups are available for covalent immobilization resulting in a decrease of multipoint covalent attachment (Mateo *et al.*, 2007). Recently, Resindion SRL has developed a new product, named Sepabeads EC-HFA, where the epoxy-groups are coupled to an ethylenediamine linker (Figure 1.16c) (Abad *et al.*, 2002; Torres *et al.*, 2003). This support (third generation) has a very high density of amino-groups as well as epoxy-groups because they

are present in a 1:1 ratio. This solves the problems of the second generation supports, while keeping all their advantages. More recently, the development of a fourth generation of epoxy-activated supports has started, which can be used in combination with engineered proteins to bind the enzymes in a specific direction (Mateo *et al.*, 2007).

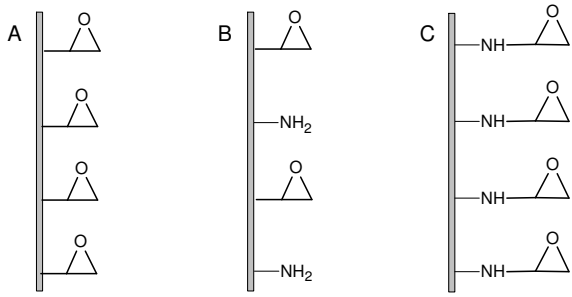

Figure 1.16 First (A), second (B) and third (C) generation epoxy-supports.

Obviously, it is crucial that the immobilized enzyme is not only stabilised but also retains as much catalytic activity as possible. Therefore, the catalytic center of the immobilised enzyme should be kept intact and accessible to the substrate (Figure 1.17). It is, however, very difficult to predict in which conformation the enzyme will be immobilized and a lot of screening (pH, T, time, concentrations, carrier types…) by trial-and-error is typically required to optimize the protocol. Immobilization of the enzyme in the presence of saturating concentrations of substrate, product or a competitive inhibitor can often reduce the occurrence of binding in unproductive conformations.

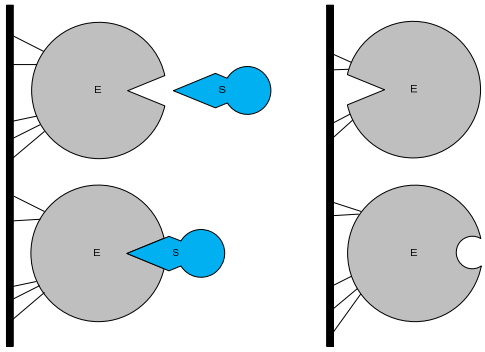

Figure 1.17 Covalent attachment of enzymes can result in different enzyme conformations (E: enzyme; S: substrate).

4.1.2 Carrier-free immobilized enzymes

Enzymes do not necessarily have to be attached to a carrier to become insoluble, but can also be crosslinked to each other with bifunctional crosslinkers, such as glutaraldehyde (Figure 1.18). In that way, the inactive mass of the carrier and the resulting 'dilution of activity' can be avoided. Enzymes, however, are highly mobile in solution and are not easy to crosslink. To overcome this obstacle, tightly-packed enzyme crystals or aggregates can be used as starting point for crosslinking (Brady & Jordaan, 2009). Such crosslinked enzyme crystals (CLEC's) or aggregates (CLEA's) have become very popular recently but only few industrial applications are reported.

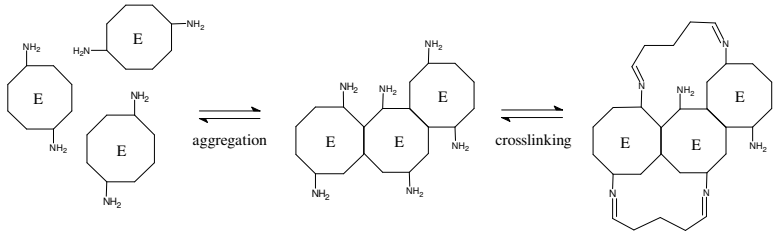

Figure 1.18 General scheme for the production of cross-linked enzyme aggregates. The enzyme molecules are first packed tightly by physical aggregation, after which chemical crosslinking can be performed.

Crosslinking of crystalline enzyme by glutaraldehyde was first described by Quiocho and Richards (Quiocho & Richards, 1964). Their main objective was to stabilize enzyme crystals for X-ray diffraction studies but they also showed that catalytic activity was retained. The use of CLEC's as industrial biocatalysts was pioneered by scientists at Vertex Pharmaceuticals in the early 1990s (Stclair & Navia, 1992) and subsequently commercialized by Altus Biologics (USA) (Margolin, 1996). CLEC's proved significantly more stable to denaturation by heat, organic solvents (Roy & Abraham, 2004) and proteolysis than the corresponding soluble enzyme. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivities, renders them ideally suited for industrial applications (Sheldon, 2007). Unfortunately, CLEC formation requires extensive protein purification and method development and only works for crystallisable enzymes (Cao *et al.*, 2003).

A less expensive method of enhancing enzyme proximity for crosslinking is by simply precipitating the protein. Brown and Glatz described in 1987 that the addition of salts (*e.g.* ammonium sulphate), organic solvents (*e.g.* *tert*-butanol) or non-ionic polymers (*e.g.* polyethylene glycol) to aqueous solutions of proteins leads to their precipitation (Brown & Glatz, 1987). The choice of the additive is important, because it can result in enzymes with slightly different three-dimensional structures without undergoing denaturation. Subsequent crosslinking

of these physical aggregates of enzyme molecules held together by non-covalent bonding, rendered them permanently insoluble and resulted in CLEA's. They were developed in Sheldon's laboratory (Cao *et al.*, 2001; Sheldon *et al.*, 2005) and commercialized by CLEA Technologies (Netherlands). The CLEA's formed exhibit comparable activities and stabilities to CLEC's (Cao *et al.*, 2000; Cao *et al.*, 2001).

Through subtle modification of the crosslinking conditions the properties of a CLEA can be adjusted significantly. Crosslinkers may be selected for optimal activity of a specific enzyme (Kaul *et al.*, 2007). Glutaraldehyde is generally the crosslinking agent of choice, since it is inexpensive and readily available in commercial quantities. The use of glutaraldehyde, however, often leads to inactivation due to its high reactivity and small size, which allows it to penetrate the internal structure of the protein where it can react with the catalytic amino acids. The use of dextran aldehyde as a crosslinker, can overcome this problem (Mateo *et al.*, 2004). A second important parameter is the duration of the precipitation step prior to crosslinking, which can change the activity and microstructure of the CLEA (Pchelintsev *et al.*, 2009). Finally, the key variable in the production of CLEA's is the crosslinking ratio, defined as the mass ratio between crosslinking agent and protein. Wilson *et al.* discovered that excess crosslinking agent reduced the enzyme conversion yield, productivity and stability and even may cause enzyme inactivation (Wilson *et al.*, 2009) while Majumder *et al.* also noted that the degree of crosslinking influenced enantioselectivity (Majumder *et al.*, 2008).

The technique is also applicable to the preparation of combi-CLEA's, containing two or more enzymes for use in one-pot, multi-step synthesis. Dalal *et al.* describe the crosslinking of a crude enzyme source, containing lipase, α-amylase and phospholipase A_2. The obtained combi-CLEA showed good multi-enzyme activity as well as stability for repeated use (Dalal *et al.*, 2007).

4.1.3 Entrapment

Entrapment of biocatalysts in an insoluble matrix does not work very well for isolated enzymes, as they often are not sufficiently retained and can show a substantial amount of leakage. However, entrapment is the method of choice to immobilize whole cells. Typically, the cells are mixed with a solution of a suitable polymer, and droplets of this mixture are then made to harden (gelation) to produce small beads that can contain up to 10 % cells. The polymers can *e.g.* be polyacrylamide or gelatine but alginate is used far most often. The latter is a linear polysaccharide consisting of uronic acids that can be crosslinked by the addition of divalent metal ions like calcium, resulting in the formation of an insoluble gel (Figure 1.19). Entrapment is relatively easy but large substrate molecules can sometimes experience difficulties in approaching the biocatalysts through the gel matrix.

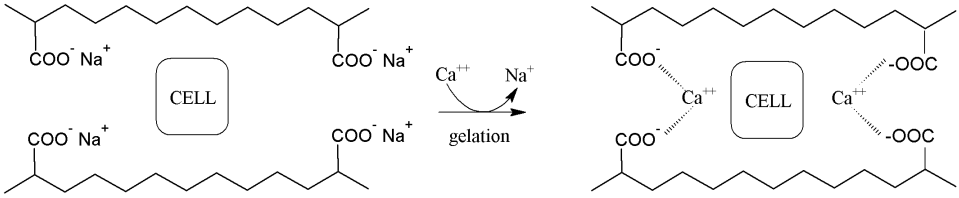

Figure 1.19 Entrapment of whole cell biocatalysts. Alginate can be crosslinked with a divalent metal ion, resulting in the formation of an insoluble gel in which the biocatalyst is trapped.

4.2 Immobilization of sucrose phosphorylase

Immobilization of sucrose phosphorylase from *L. mesenteroides* was first described in 1977 by Grazi *et al.* where it was embedded in fibres of cellulose triacetate (Table 1.6) (Grazi *et al.*, 1977). At least 40 % of the original activity was recovered when the enzyme was entrapped in the fibres. The immobilized biocatalyst was active in a broader pH range and could be stored for months at 2 °C without loss of activity. At pH 6.0 and 37 °C, the immobilized SP could be used continuously and repeatedly for days without loss of activity, indicating the good operational stability of the fibre-entrapped enzymes.

Taylor *et al.* prepared immobilized SP from *Pseudomonas saccharophila* by covalent grafting onto porous ceramic beads (Taylor *et al.*, 1982). The enzyme maintained about 30 % of the activity after immobilization and there was no diffusional resistance determined to the enzyme-catalyzed reaction. The half-life of the immobilized enzyme varied from about 35 days at 30 °C to about 5 days at 40 °C.

Guibert & Monsan reported an operational half-life of 3.5, 22 and 220 days at 45 °C, 40 °C and 30 °C, respectively, for partially purified SP from *L. mesenteroides* immobilized onto corn stover (Guibert & Monsan, 1988). Unfortunately, these authors did not describe binding efficiencies for that carrier.

In 1991, immobilization of SP from *L. mesenteroides* by covalent linkage to several supports, and by adsorption onto DEAE-cellulose, was reported (Pimentel & Ferreira, 1991). Covalent attachment onto polyethyleneimine, polyacrylamide beads and CM-cellulose azide retained, respectively, 5, 11 and 2 % of the specific activity. The polyacrylamide bound enzyme was the less stable derivative, losing all activity after 24 h of discontinuous use. Although immobilization onto DEAE-cellulose retained only 18 % of specific activity, it was the most stable. During 6 days of utilization at 40 °C, the absorbed enzyme lost 40 % of maximum activity and 20 % of the

total absorbed proteins. Afterward, the preparation remained stable to batch discontinuous use at pH 7.0 and 40 °C for about 8 months.

In another study, the cells of *L. mesenteroides* were entrapped in gelatine beads. The obtained immobilized SP exhibited good mechanical stability and a half-life of 40 days at 30 °C (Soetaert *et al.*, 1995).

Goedl *et al.* described the covalent attachment of SP from *L. mesenteroides* onto Eupergit C with a binding efficiency of approximately 50 % and provided a very stable immobilized enzyme preparation (Goedl *et al.*, 2007). To obtain a recyclable biocatalytic carrier, also non-covalent attachment of SP onto anion-exchange materials such as EMD-DEAE (S) and Q-Sepharose was examined. However, low binding efficiency (< 15 %) and significant leaching of the enzyme under operational conditions dismissed these carriers for further consideration. The immobilized biocatalyst was used for the continuous production of α-D-glucose-1-phosphate from sucrose and phosphate (600 mM each) in a fixed bed reactor (30 °C, pH 7.0). The reactor was operated at a stable conversion of 91 % and productivity of approximately 11 g/L/h for up to 650 h, emphasising the excellent stability of immobilized SP under the operational conditions.

Although very interesting, none of these immobilized enzyme preparations allow processes to be performed at 60 °C, the temperature required for carbohydrate conversions at the industrial scale. Consequently, there still is a clear need to improve the thermal stability of sucrose phosphorylase, which is the topic of this PhD thesis.

Table 1.6 Overview of immobilized sucrose phosphorylases

Technique	Strain	Y_{immo}	Operational stability[b]	References
Entrapment in cellulose triacetate fibres	*L. mesenteroides*	≥ 40 %	days (37 °C)	Grazi *et al.*, 1977
Covalent attachment on porous ceramic beads	*P. saccharophila*	30 %	$t_{1/2}^{30}$ = 35 d $t_{1/2}^{40}$ = 5 d	Taylor *et al.*, 1980
Covalent grafting on corn stover	*L. mesenteroides*	120 U/g	$t_{1/2}^{30}$ = 220 d $t_{1/2}^{40}$ = 22 d $t_{1/2}^{45}$ = 3.5 d	Guibert & Monsan, 1988
Covalent attachment[a] Adsorption on DEAE-cellulose	*L. mesenteroides*	≤ 11 % ≤ 18 %	< 24 h[c] ~8 months (40 °C)	Pimentel & Ferreira, 1991
Entrapment in gelatin beads	*L. mesenteroides*	-	$t_{1/2}^{30}$ = 40	Soetaert *et al.*, 1995
Covalent attachment on carrier	*L. mesenteroides*	50 %	>27 d (30 °C)	Goedl *et al.*, 2007

[a] on nylon polyethyleimine, polyacrylamide beads and CM-cellulose azide; [b] in presence of sucrose; [c] temperature not reported.

CHAPTER 2

ENZYME EXPRESSION AND CHARACTERIZATION

1. INTRODUCTION

To date, sucrose phosphorylase (SP) has been identified in only a relatively small number of bacterial species, none of which are thermophilic (Silverstein *et al.*, 1967; Russell *et al.*, 1988; van den Broek *et al.*, 2004; Lee *et al.*, 2006). As carbohydrate conversions need to be operated at 60 °C or higher to avoid microbial contamination, the lack of a thermostable enzyme is a major limitation of the industrial application of SP (Vieille & Zeikus, 1996; Eijsink *et al.*, 2005). Consequently, the goal of this PhD thesis is to increase its thermostability by various engineering strategies. To that end, SP has first been recombinantly expressed and characterized.

Two SP enzymes have been selected as template for engineering, *i.e.* that from *L. mesenteroides* (*Lm*SP) and that from *B. adolescentis* (*Ba*SP). The former was chosen because it has been used for the majority of reported glycosylation reactions (Goedl *et al.*, 2010) and because our laboratory has acquired extensive expertise with it (Vandamme *et al.*, 1987). The latter was chosen because its crystal structure is available (Sprogoe *et al.*, 2004) and because it exhibits the highest temperature optimum (48 °C) reported so far (van den Broek *et al.*, 2004).

In this chapter, the heterologous expression of *Lm*SP and *Ba*SP in *E. coli* will first be described. As recombinant expression has to meet certain requirements, especially when it's followed by high-throughput screening, the protocols have been thoroughly optimized. In particular, a constitutive expression system has been developed that should drastically increase the throughput and accuracy of the screening process, since it eliminates the need for adding an inducer to the cultures.

High-throughput screening also requires the availability of a convenient assay procedure that is both accurate and inexpensive. The most common method to measure SP activity is a continuous coupled enzymatic assay that allows the monitoring of NADH formation at 340 nm (Silverstein *et al.*, 1967; Weinhausel *et al.*, 1997). Although very accurate, this assay is far from cheap. Therefore, a practical alternative is presented here that is based on the detection of reducing sugars in a simple colorimetric assay employing inexpensive chemicals.

Finally, both SP enzymes have been purified and fully characterized with respect to their kinetic parameters, pH and temperature optimum. Most crucial is the determination of the enzymes' stability at various temperatures, as this will provide us with a point of reference for the engineering experiments described in the next chapters.

2. MATERIALS AND METHODS

2.1 Microbial strains, growth conditions and chemicals

L. mesenteroides ATCC12291 and *B. adolescentis* LMG10502^T were obtained from the American Type Culture Collection (ATTC) and the Belgian Co-ordinated Collections of Micro-organisms (BCCM), respectively. *L. mesenteroides* was grown at 30 °C in MRS broth (Oxoid) supplemented with 10 % (v/v) of a vitamin solution containing 0.1 % $FeSO_4.7\ H_2O$, 2 % $MnSO_4.H_2O$, 0.1 % thiamine.HCl, 0.5 % ascorbate and 0.3 % citrate. *B. adolescentis* was grown anaerobically at 37 °C in a medium containing 2.3 % special peptone (Oxoid), 0.1 % soluble starch, 0.5 % NaCl, 0.03 % cysteine hydrochloride and 0.5 % glucose.

E. coli XL10-Gold (Stratagene) was used as cloning strain, while *E. coli* Rosetta 2 (Novagen) was used for recombinant expression of SP genes. Competent *E. coli* cells were prepared according to the Inoue method (Sambrook & Russell, 2001). Transformed *E. coli* was routinely grown at 37 °C and 200 rpm in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 0.1 g/L ampicillin.

Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Oligonucleotides were synthesized by Sigma. Kits for gel extraction, PCR purification and plasmid isolation were purchased from Qiagen. Sequencing of the obtained expression plasmids was performed by LGC Genomics. All chemicals were obtained from Sigma unless otherwise stated.

2.2 Construction of expression vectors

2.2.1 Inducible expression (Figure 2.1)

Genomic DNA (gDNA) was extracted from overnight cultures with the 'GenElute Bacterial Genomic DNA kit' from Sigma, using the protocol for Gram-positive bacteria. The *Lm*SP and *Ba*SP genes were amplified from the gDNA by high fidelity PCR with the primer pairs listed in Table 2.1. The PCR reactions contained 25 ng gDNA, 2.5 U *Pfu*Ultra DNA polymerase (Stratagene), 10x *Pfu*Ultra HF AD buffer, 0.2 mM of dNTP mix (Westburg) and 0.4 µM of each primer in a total volume of 25 µL. The high GC content of the gDNA of *B. adolescentis* (= 60 %) required the addition of DMSO to the PCR mixture to a final concentration of 5 %. PCR cycling conditions are shown in Table 2.2.

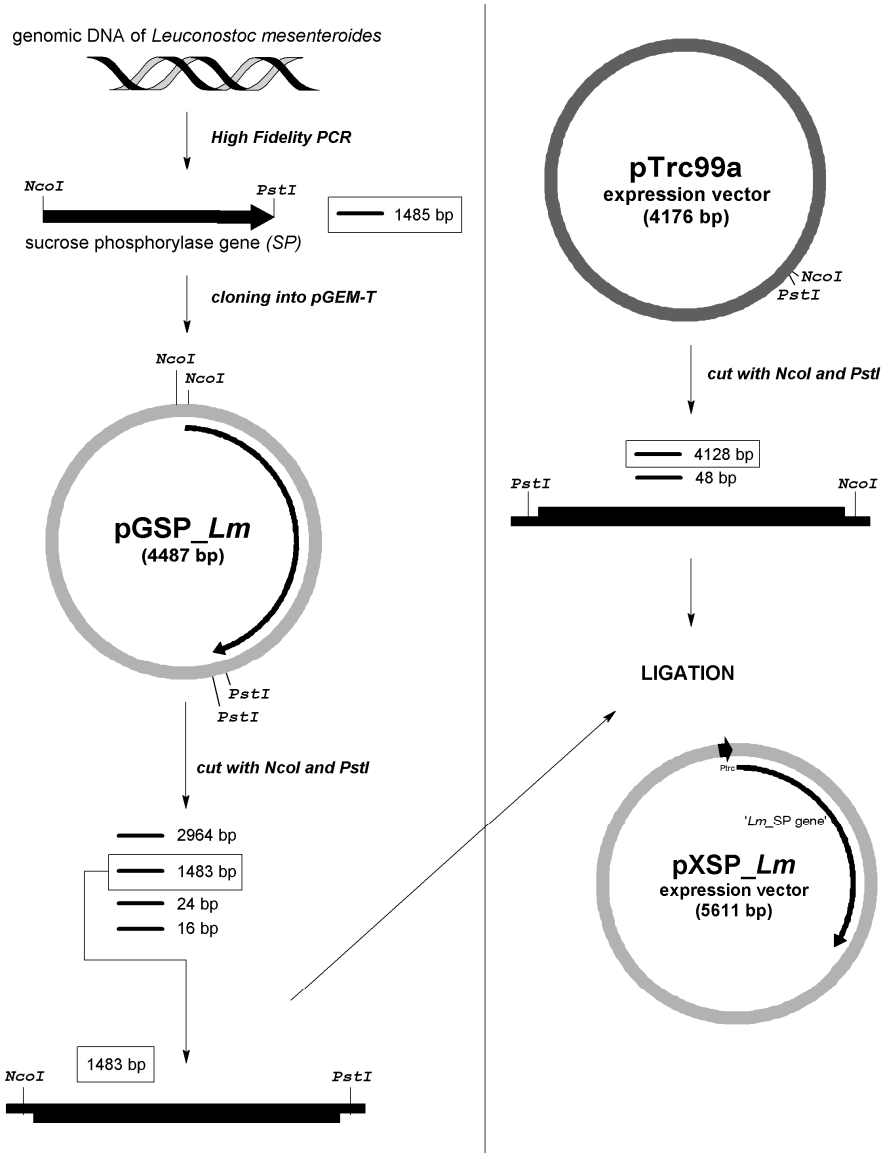

Figure 2.1 Cloning strategy for the construction of the inducible pXSP_*Lm* expression vector.

Table 2.1 Primers for the amplification of *Lm*SP and *Ba*SP genes

Gene	Primer	Sequence (5'→3')	Restriction site
*Lm*SP	Forward	CCATGGATGGAAATTCAAAACAAAGCAATG	NcoI
	Reverse	CTGCAGTTAGTTCTGAGTCAAATTATCACTGC	PstI
*Ba*SP	Forward	CCATGGATGAAAAACAAGGTGCAGCTCATC	NcoI
	Reverse	TCACTGCAGTCAGGCGACGACAGGCGGATT	PstI

A 1485 bp and 1530 bp fragment containing the full-length *Lm*SP and *Ba*SP gene, respectively, was thus obtained and ligated into the pGEM-T vector (Promega). The resulting plasmids were named pGSP_*Lm* and pGSP_*Ba*, respectively, and were used to construct the expression vectors for *Lm*SP and *Ba*SP. After digestion of these two plasmids with NcoI and PstI (in the same reaction), the correct fragments were ligated into the predigested inducible pTrc99A vector with NcoI and PstI. After an overnight incubation at 22 °C, *E. coli* was transformed with the ligation mixtures and plated on LB medium supplemented with ampicillin. The SP expression vectors obtained after plasmid extraction were named pXSP_*Lm* and pXSP_*Ba*.

Table 2.2 PCR cycling conditions

Cycle	*Lm*SP		*Ba*SP	
1 x	3 min	94 °C	10 min[a]	95 °C
35 x	1 min	94 °C	1 min	94 °C
	1 min	50 °C	1 min	55 °C
	3 min	72 °C	3 min	72 °C
1 x	7 min	72 °C	7 min	72 °C

[a] polymerase added after 8 min

2.2.2 Constitutive expression

Genomic DNA was extracted from overnight cultures with the 'GenElute Bacterial Genomic DNA kit' from Sigma, using the protocol for Gram-positive bacteria. The *Lm*SP and *Ba*SP genes were amplified from the genomic DNA using the same protocol as described for the inducible expression system albeit with different primer pairs (Table 2.3).

A 1487 bp and 1532 bp fragment containing the full-length *Lm*SP and *Ba*SP gene, respectively, was obtained, digested with the proper restriction endonucleases and ligated into various pCXPXXh vectors (Aerts *et al.*, 2010) predigested with NheI and PstI. After an overnight incubation at 22 °C, *E. coli* was transformed with the ligation mixtures and plated on LB medium supplemented with ampicillin. The constitutive SP expression vectors obtained after plasmid extraction were named pCXPXXh_*Lm*SP and pCXPXXh_*Ba*SP, respectively.

Table 2.3 Primers for the amplification of *Lm*SP and *Ba*SP genes

Gene	Primer	Sequence (5'→3')	Restriction site
*Lm*SP	Forward	TAGCTAGCATGGAAATTCAAAACAAAGC	NheI
	Reverse	CTGCAGTTAGTTCTGAGTCAAATTATCACTGC	PstI
*Ba*SP	Forward	TAGCTAGCATGAAAAACAAGGTGCAGCTCATC	NheI
	Reverse	TCACTGCAGTCAGGCGACGACAGGCGGATT	PstI

2.3 Optimization of recombinant SP expression

The constructed expression vectors were transformed into *E. coli* Rosetta 2 and a single colony from each transformation was picked and grown overnight at 37 °C in 5 mL LB medium supplemented with ampicillin. The overnight culture was used as inoculum (2 % v/v) for an enzyme expression culture. Once the expression cultures reached the exponential phase (OD_{600} ~ 0.6), the cultures were further grown at different temperatures and at that time IPTG was added in different concentrations to the inducible expression system. At different times, 1 mL samples of the culture were centrifuged for 5 min at 18 000 rpm and the obtained pellets were frozen at -20 °C.

Crude cell preparations were prepared by chemo-enzymatic lysis of *E. coli* pellets containing recombinantly expressed enzyme. Frozen pellets were thawed at room temperature and suspended in 200 µL of lysis solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 50 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1mg/mL lysozyme. After a 5 min incubation at room temperature, the lysis mixture was centrifuged at 18 000 rpm and the supernatant was transferred to a new tube. The obtained crude cell preparations were tested for SP activity using a continuous coupled enzymatic assay in which the release of α-D-glucose-1-phosphate was measured as described in the next section.

2.4 Evaluation of activity assays

Different assays to determine the activity of SP were compared. The first is a continuous coupled enzymatic assay, in which production of αG1P from sucrose and inorganic phosphate is coupled to the reduction of NAD^+ in presence of phosphoglucomutase (PGM) and glucose-6-phosphate dehydrogenase (G6P-DH). The standard assay solution contains 100 mM Tris-HCl (pH 7.0), 2 mM EDTA, 10 mM $MgSO_4$, 2 mM β-NAD, 10 µM glucose-1,6-diphosphate, 1.2 U PGM and 1.2 U G6P-DH (Silverstein *et al.*, 1967; Weinhausel *et al.*, 1997). As substrate, 100 mM sucrose in 100 mM phosphate buffer, pH 7.0, was used. The absorbance was measured at 340 nm in a microtiter plate reader equilibrated at 37 °C. Alternatively, the assay was performed in a

discontinuous way by inactivation samples by heating (95 °C) at regular intervals. A third method is measuring the release of fructose with the discontinuous bicinchoninic acid (BCA) assay (Waffenschmidt & Jaenicke, 1987). One unit (U) of SP activity corresponds to the release of 1 µmole product per minute from 100 mM sucrose in 100 mM phosphate buffer under the described conditions. All activity assays were performed in triplicate and had a coefficient of variance (CV) of less than 10 %. The protein concentration was measured in triplicate with the BCA Protein assay (Pierce), using bovine serum albumin (BSA) as standard.

2.5 Determination of optimum pH and temperature

The influence of the pH on the phosphorolytic activity of SP was studied at 37 °C in McIlvaine buffers (McIlvaine, 1921) of different pH values (4.5-8.0) containing saturated concentrations of both substrates. The influence of temperature was measured between 30 and 70 °C in 100 mM phosphate buffer, pH 7.0. Activities were measured with the BCA assay.

2.6 Kinetic parameters for sucrose

Kinetic parameters were determined at the optimal temperature and pH in 100 mM phosphate buffer containing varying concentrations of sucrose, using the discontinuous BCA assay. The kinetic parameters were obtained from non-linear fits of the Michaelis–Menten equation using the program Sigmaplot for Windows version 10.0.

2.7 Enzyme production for purification

E. coli cells transformed with the constitutive expression plasmids pCXP34h_*Lm*SP and pCXP14h_*Ba*SP were cultivated in 1 L shake flasks at 37 °C using LB medium supplemented with 0.1 g/L ampicillin. Once the expression cultures reached the exponential phase (OD_{600} ~ 0.6), the recombinant proteins were further expressed at 25 °C and 37 °C, respectively. After 6 hours, the cells were harvested by centrifugation (7 000 rpm, 4 °C, 20 min) and the obtained pellets were frozen overnight at -20 °C. Frozen pellets were thawed, suspended in lysis solution (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mg/mL lysozyme (Roche) and 0.1 mM, PMSF, pH 8.0) and disrupted by sonication for 2 x 2 min (Branson 250 Sonifier, level 3, 50 % duty cycle). Cell debris was removed by centrifugation (15 000 rpm, 4 °C, 30 min). The N-terminal His_6-tagged protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography, as described by the supplier (Qiagen). Buffer exchange to 100 mM phosphate

buffer, pH 7.0, was achieved with a centricon in order to remove the imidazole present in the elution buffer.

2.8 Thermostability

Purified *Lm*SP and *Ba*SP enzyme were diluted in 100 mM phosphate buffer at pH 6.8 and 6.5, respectively, and incubated at different temperatures in a Thermoblock (Stuart SBH130D). At certain times, samples were cooled on ice and the remaining activity was determined using the BCA method. The influence of the SP concentration on the thermal stability was determined by inactivating different concentrations of enzyme. Thermostability assays were performed in triplicate and had a CV of less than 10 %.

3. RESULTS AND DISCUSSION

3.1 Construction of expression vectors

3.1.1 Inducible expression

High fidelity PCRs on the genomic DNA of *L. mesenteroides* and *B. adolescentis* resulted in fragments of the expected length for the *Lm*SP and *Ba*SP genes, *i.e.* 1485 and 1530 bp, respectively (Figure 2.2a). After cloning of the PCR fragments into pTrc99a, as described in the Materials and methods section, the expression vectors pXSP_*Lm* and pXSP_*Ba* were obtained and checked by sequencing. A schematic representation of the pXSP_*Lm* vector is shown in Figure 2.2b.

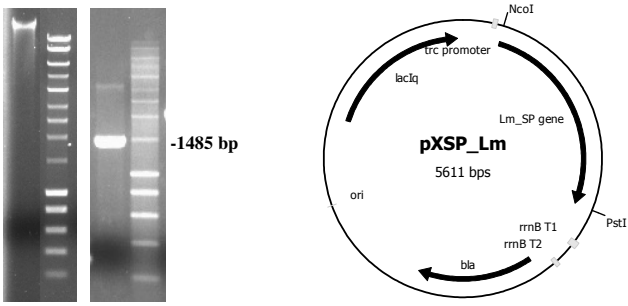

Figure 2.2 A. Results of genomic DNA extraction of *Lm*SP (left) and PCR on *Lm*SP (right) B. The inducible pXSP_*Lm* expression vector.

3.1.2 Constitutive expression

For the construction of the constitutive expression plasmids, a similar amplification protocol was used albeit with different primer pairs (Table 2.3). Then, the obtained full-length *Lm*SP and *Ba*SP genes were cloned into the pCXPXXh vectors. These vectors were constructed in the laboratory and made up a set of four constitutive expression plasmids with promoters (PXX) of varying strengths (Aerts *et al.*, 2010). These promoters were selected from a library of 57 artificial constitutive promoters for *E. coli*, which were constructed by De Mey and colleagues (De Mey *et al.*, 2007). The strength of the synthetic promoters was determined by expressing green fluorescent protein (GFP) in *E. coli* MA8 cells and measuring the relative fluorescence units (Figure 2.3).

The construction of the pCXPXXh plasmid set allows the constitutive expression of *Lm*SP and *Ba*SP with a N-terminal 6x His-tag, under control of the promoters P14, P34, P22 and P78, listed in order of decreasing strength (Figure 2.3) (Aerts *et al.*, 2010). As the strength of the promoter can have a very big influence on the amount of soluble protein, this plasmid set was used to minimize the formation of inclusion bodies during the expression of SP. The availability of a constitutive expression system would also facilitate high throughput screening.

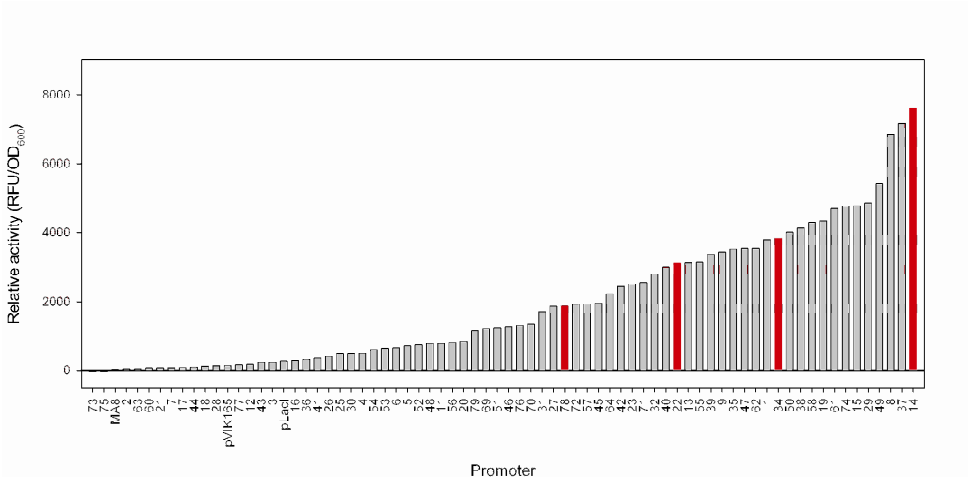

Figure 2.3 Library of artificial promoters for *E. coli*. Promoter activity was assayed for a reporter gene encoding GFP, transcribed from the synthetic promoter clones (De Mey *et al.*, 2007).

3.2 Optimization recombinant expression

To determine whether a constitutive expression system can compete with a conventional inducible system, the expression of the SP enzymes was optimized for both systems. All SP expression vectors were transformed into *E. coli* Rosetta 2. This strain, which supplements tRNAs for rare codons, was chosen as host strain to prevent difficulties in expression due to the presence of rare codons in the SP genes.

The inducible expression vectors pXSP_*Lm* and pXSP_*Ba* carry the *lacI*^{q} gene and contain the strong synthetic *trc* promoter. The expression of the SP enzymes was optimized using different IPTG concentrations (0 – 2 mM), induction times (0 – 6 hours) and induction temperatures (25, 30 and 37 °C). The optimal conditions of the expression of *Lm*SP and *Ba*SP was found to be 2 and 6 hours induction with 0.1 and 0.5 mM IPTG at 25 and 37 °C, respectively (Figure 2.4 and Figure 2.5). An activity of 1.1 and 1.5 U/mL cell extract, respectively, was then obtained.

The constitutive expression plasmid sets pCXPXXh_*Lm* and pCXPXXh_*Ba* comprise four different promoters of varying strength. The availability of these two plasmid sets facilitates the optimization of the constitutive expression of *Lm*SP and *Ba*SP. For enzymes that are not toxic and not tending to form inclusion bodies, a strong constitutive promoter may be desirable. In contrast, intermediate and weak constitutive promoters are advisable for enzymes whereof soluble expression is difficult to achieve. Besides the promoter strength, the expression time (0 – 6 hours) and temperature (25, 30 and 37 °C) were also optimized.

For the constitutive expression of *Lm*SP, the temperature appeared to be crucial, in comparison with *Ba*SP where this is not of significant importance. At 37 °C, only a very low activity could be observed and this in similarity with the inducible expression system. By lowering the expression temperature, the growth was reduced but the obtained activity increased. This was in accordance with the higher activities measured when intermediate promoter strengths were used, implying reduced formation of inclusion bodies. Thus, the optimal conditions for constitutive expression of *Lm*SP were 6 hours expression at 25 °C with the intermediate promoter strength of P34, yielding similar expression levels as for the inducible expression system. The residual activities of *Lm*SP expressed with the different promoters are shown in Figure 2.4 for its optimal temperature of 25 °C.

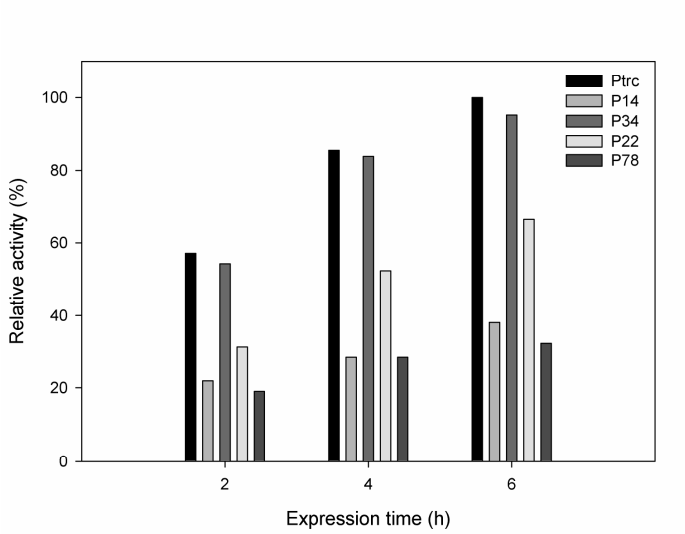

Figure 2.4 Relative activities of *Lm*SP at different expression times with different promoters at the optimal temperature of 25 °C. After chemo-enzymatic cell lysis, activity assays were performed by measuring the production of αG1P from 100 mM sucrose and 100 mM phosphate at pH 7.0 and 37 °C.

For *Ba*SP, the maximum activity was obtained after 6 hours of expression with the strongest promoter P14 at 37 °C and was only 8.2 % less than the maximal activity obtained using the inducible expression system. For both enzymes, the maximal activity was reached at the end of the exponential phase. The results of the *Ba*SP expression with different promoter strengths are displayed in Figure 2.5 for its optimal expression temperature of 37 °C.

These experiments show that constitutive expression of SP is a practical alternative for the inducible system, resulting in similar expression levels while eliminating a pipetting step. Therefore, this strategy will be used for the production of SP in the remainder of this work.

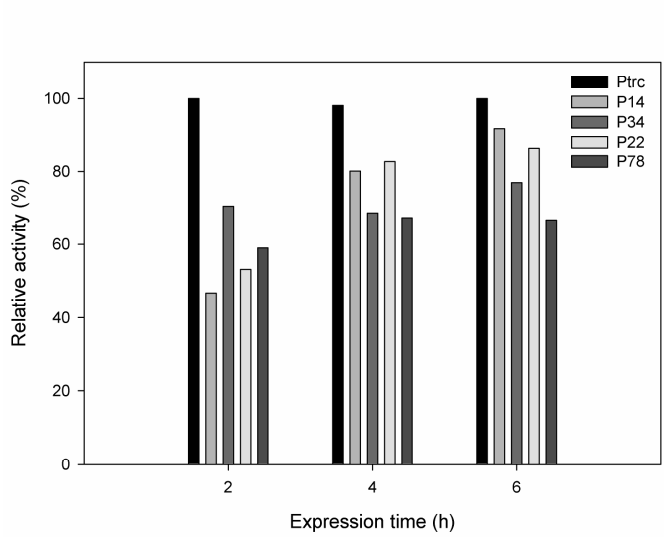

Figure 2.5 Relative activities of *Ba*SP at different expression times with different promoters at the optimal temperature of 37 °C. After chemo-enzymatic cell lysis, activity assays were performed by measuring the production of αG1P from 100 mM sucrose and 100 mM phosphate at pH 7.0 and 37 °C.

3.3 Production and purification of recombinant His-tagged SP

In order to determine the enzymatic properties of SP, His-tagged *Lm*SP and *Ba*SP were purified. To that end, *E. coli* cells were transformed with the constitutive expression vectors pCXP34h_*Lm*SP and pCXP14h_*Ba*SP. After chemo-enzymatic cell lysis, a crude enzyme preparation was obtained with a specific *Lm*SP and *Ba*SP activity of approximately 20 and 29 U/mg, respectively. The recombinant SP enzyme carries a N-terminal fusion peptide with the sequence Gly-Gly-Ser-His_6-Gly-Met-Ala-Ser that provides metal-binding affinity. SP could therefore be purified by affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) metal matrix. The purified biocatalyst migrated as a single protein band in Coomassie-stained SDS-PAGE with the calculated molecular mass of the His-tagged enzyme being 57.2 and 57.7 kDa for *Lm*SP and *Ba*SP, respectively (Figure 2.6).

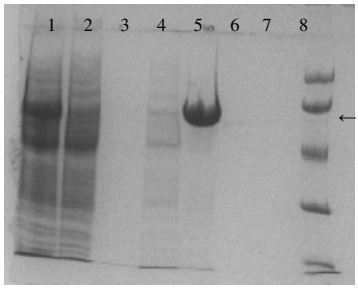

Figure 2.6 His-tag purification of *Ba*SP. Lane 1: cleared lysate; lane 2: flow through; lane 3-4: wash 1 & 2; lane 5: purified enzyme; lane 6-7: flow through centricon, lane 8: LMW standard.

After buffer exchange in a centricon to remove the possibly denaturing imidazole, a purification yield of 45 % and a 5.5-fold increase in specific activity (to 161 U/mg) could be determined for *Ba*SP. The purification scheme of *Ba*SP is shown in Table 2.4. Similar results were obtained for the purification of SP from *L. mesenteroides*. It has to be mentioned that the final specific activity can differ from batch to batch, e.g. for *Ba*SP specific activities between 160 and 210 U/mg were found. The variation in expression between different batches makes it sometimes difficult to predict the optimal resin/protein ratio. Too much resin can cause nonspecific binding and if too little resin is used, some of the protein will be washed away, both resulting in a lower purification efficiency. Typically 0.5 mL resin was used to purify 250 mL culture.

Table 2.4 Purification scheme of *Ba*SP

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Cleared lysate	178.5	5219	29	-	1
Purified enzyme	14.4	2333	161	45	5.5

3.4 Evaluation of activity assays

SP activity is traditionally determined using a continuous enzymatic assay, in which the production of αG1P from sucrose and inorganic phosphate is coupled to the reduction of NAD^+ in the presence of PGM and G6P-DH (Silverstein *et al.*, 1967; Weinhausel *et al.*, 1997) (Figure 2.7).

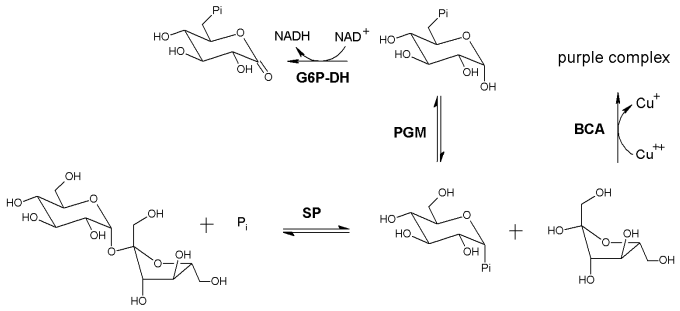

Figure 2.7 Different assays for the determination of SP activity.
BCA, bicinchoninic acid; PGM, phosphoglucomutase; G6P-DH, glucose-6-phosphate dehydrogenase.

However, we have found that this continuous assay is not suitable for the determination of kinetic parameters. A systematic underestimation of the activity is observed and the difference increases exponentially with increasing substrate concentrations (Figure 2.8). This difference is probably caused by the kinetic parameters of the enzymes involved in the αG1P determination. Therefore, the assay has to be performed discontinuously, taken samples at regular intervals. In that case, the assay can also be replaced by the discontinuous BCA assay, which measures the release of the reducing sugar fructose from the non-reducing substrate sucrose. Comparison of both discontinuous assays has shown that BCA is much more sensitive, implying that less enzyme has to be added to the reaction. However, comparable kinetic parameters could be obtained, i.e. K_m values of 6.8±1.2 and 6.3±1.1 mM, respectively for *Ba*SP. The BCA method is thus a very reliable alternative for the discontinuous αG1P assay. Furthermore, the composition of the solution is much cheaper and the assay is performed in the visible spectrum, avoiding the need of expensive UV-transparent microtiter plates.

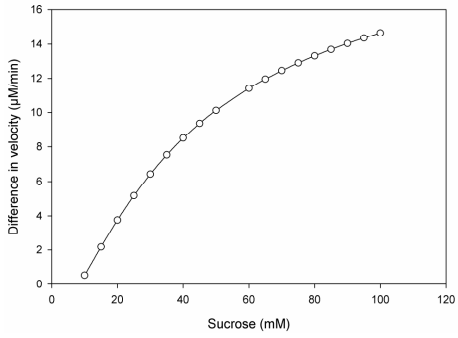

Figure 2.8 The difference in activity of *Lm*SP when using the continuous versus discontinuous αG1P assay.

3.5 Characterization of the recombinant SP

Both purified SP enzymes were characterized in order to examine their thermostability. Furthermore, the pH en temperature profile as well as the kinetic parameters were also determined.

3.5.1 pH and temperature profile

First, the optimal pH and temperature for phosphorolytic activity were determined. Both enzymes were found to have a rather narrow pH profile with optima of 6.8 and 6.5 for *Lm*SP and *Ba*SP, respectively (Figure 2.9). Although these pH optima correspond well with previously reported values (van den Broek *et al.*, 2004; Lee *et al.*, 2006), a complete pH profile was not yet available for *Ba*SP. Other phosphorolytic enzymes also display maximal activity near neutral pH (Kitaoka *et al.*, 1992; Huwel *et al.*, 1997; Eis & Nidetzky, 1999).

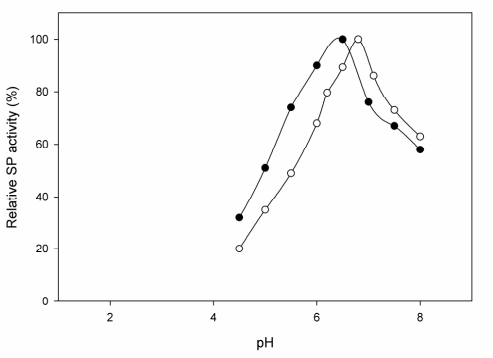

Figure 2.9 pH profile of purified *Lm*SP (○) and *Ba*SP (●). Phosphorolysis reactions were performed at 37 °C in McIlvaine buffer (pH 4.5 – 8) using saturated concentrations of both substrates.

Interestingly, the optimal temperature for *Ba*SP is 16 degrees higher than that for *Lm*SP, with temperature optima of 58 and 42 °C, respectively (Figure 2.10). At 58 °C, *Lm*SP only has a remaining activity of less than 30 %. This high temperature optimum for *Ba*SP is in contrast with the value of 48 °C that has been reported for the SP without His-tag (van den Broek *et al.*, 2004). For SP from *L. mesenteroides*, however, it has been shown that the introduction of a His-tag does not influence the enzyme's properties (Lee *et al.*, 2006; Goedl *et al.*, 2007). Therefore, it can be assumed that this increased optimal temperature is not caused by the His-tag but rather by differences in assay procedures. The high optimal temperature of SP from *B. adolescentis* indicates potential stability at elevated temperatures.

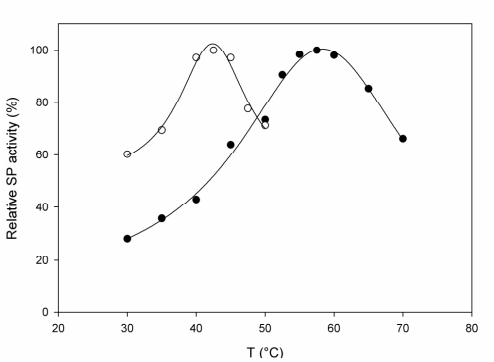

Figure 2.10 Temperature profile of purified *Lm*SP (○) and *Ba*SP (●).
SP activity was measured in 100 mM phosphate buffer, pH 7.0.

3.5.2 Kinetic parameters

The kinetic parameters for the phosphorolytic reaction were determined at optimal pH and temperature by measuring the release of reducing sugar (fructose) using the BCA method. The kinetic parameters K_m and k_{cat} of *Lm*SP for sucrose have been found to be 23.2±2.6 mM and 44±5.6 s^{-1}, respectively. For *Ba*SP, however, the K_m and k_{cat} for sucrose are 6.8±1.2 mM and 207±17 s^{-1}, respectively (Table 2.5). Thus, the *Ba*SP enzyme presents a higher affinity for its substrate sucrose and also converts more sucrose molecules per time unit than the *Lm*SP enzyme.

Table 2.5 Characteristics of *Lm*SP and *Ba*SP

Characteristics	*Lm*SP	*Ba*SP
pH_{opt}	6.8	6.5
T_{opt} (°C)	42	58
K_m (mM)	23.2±2.6	6.8±1.2
k_{cat} (s^{-1})	44±5.6	207±17

3.5.3 Thermostability

Because of the large difference in optimal temperature of both SP enzymes, it was interesting to examine whether this was also reflected in their thermostability. At the optimal temperature of *Ba*SP (58 °C), *Lm*SP only shows less than 30 % of its maximal activity. Therefore, *Lm*SP is not likely to be as stable as *Ba*SP. The thermostability was determined with purified *Lm*SP and *Ba*SP solutions of 0.46 U/mL in 100 mM phosphate buffer at pH 6.8 and 6.5, respectively. The effect

of the temperature on the stability of *Lm*SP and *Ba*SP are displayed in Figure 2.11 and Figure 2.12, respectively.

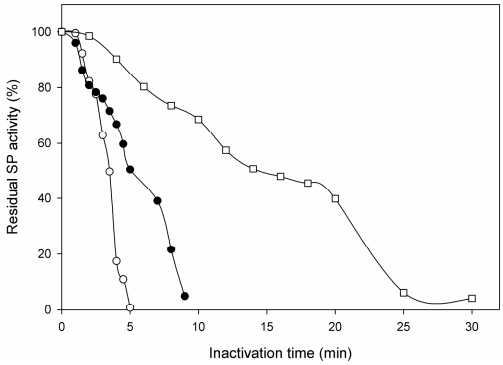

Figure 2.11 Effect of temperature on the stability of purified *Lm*SP. Incubation at 50 °C (□), 55 °C (●) and 60 °C (○) in 100 mM phosphate buffer, pH 6.8 using 0.46 U/mL SP enzyme in the reaction mixture.

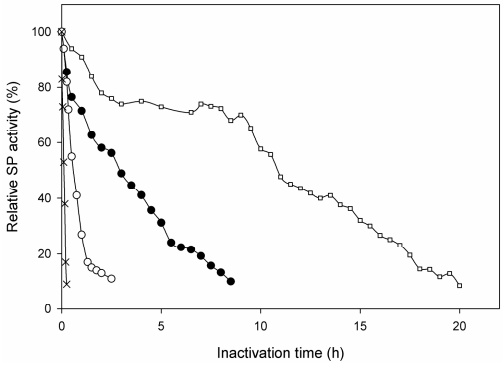

Figure 2.12 Effect of temperature on the stability of purified *Ba*SP. Incubation at 60 °C (□), 65 °C (●), 70 °C (○) and 75 °C (×) in 100 mM phosphate buffer, pH 6.5 using 0.46 U/mL SP enzyme in the reaction mixture.

The SP from *B. adolescentis* clearly presents a higher temperature tolerance compared to the enzyme from *L. mesenteroides*. At 60 °C, *Ba*SP still retains 60 % of its initial activity after 10 hours incubation, whereas *Lm*SP loses all its activity already after 5 min incubation. Table 2.6 gives an overview of the half-life times ($t_{1/2}$) of denaturation, defined as the time it takes for the

activity of the protein to be reduced by half at defined temperatures (kinetic stability). These values show again the large difference in thermostability between both SP enzymes.

Table 2.6 Half-live times ($t_{1/2}$) of *Lm*SP and *Ba*SP.

Temperature (°C)	$t_{1/2}$ of *Lm*SP (min)	$t_{1/2}$ of *Ba*SP (min)
50	14	-[a]
55	5	-
60	3.5	660
65	-	180
70	-	30
75	-	7

[a] - = not determined

As SP from *B. adolescentis* is the most promising target for increasing the thermostability of sucrose phosphorylases, its thermostability was examined in more detail. Initial experiments with the crude enzyme preparation indicated that *Ba*SP retained more than 70 % of the initial activity when incubated in phosphate buffer pH 6.5 for 30 min at 70 °C. In contrast, Figure 2.12 shows that the purified enzyme preparation (0.46 U/mL) only retained 50 % of its activity under those conditions. This difference suggests that SP may be stabilized by effectors present in the crude enzyme preparation. Furthermore, the stability of the purified *Ba*SP has been found to strongly depend on the enzyme concentration. Figure 2.13 shows that higher *Ba*SP concentrations tend to stabilize the enzyme and the same trend was observed for *Lm*SP. In literature, this phenomenon has occasionally been reported, for example for α-L-fucosidase (Dicioccio *et al.*, 1983). In the case of crude enzyme preparations almost no effect of the SP concentration was observed within the range of 0.046 and 4.6 U/mL.

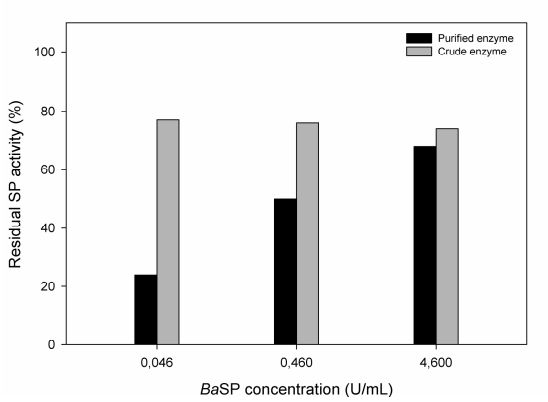

Figure 2.13 Effect of protein concentration on the thermostability of purified and crude *Ba*SP preparations: 30 min incubation at 70 °C in 100 mM phosphate buffer, pH 6.5 using different *Ba*SP concentrations in the reaction mixture.

Further thermostability tests on *Ba*SP were carried out at 60 °C, which is the optimal temperature for carbohydrate conversions in industry. Varying concentrations of purified enzyme were incubated overnight, after which residual SP activities were measured. Figure 2.14 revealed again that increasing the protein concentration results in better thermostability. The maximum residual SP activity that could be reached was 80 % after 16 hours incubation at 60 °C, requiring an enzyme concentration of 40 U/mL.

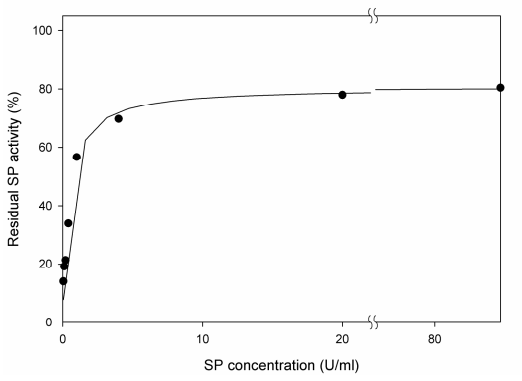

Figure 2.14 Effect of protein concentration on the thermostability of purified *Ba*SP enzyme: overnight incubation at 60 °C in 100 mM phosphate buffer, pH 6.5 using different *Ba*SP concentrations in the reaction mixture.

4. CONCLUSIONS

The results discussed above show that both inducible and constitutive vectors can successfully be used for the expression of *Lm*SP and *Ba*SP. Under optimal conditions, both expression systems yield similar activity levels of approximately 1 and 1.5 U/mL, respectively. As constitutive expression is much more practical, this strategy will be used for future protein engineering experiments. In that way, the high throughput screening can be facilitated by circumventing the induction step and minimizing the amount of consumables. Furthermore, a new assay has been developed for SP, which is optimally suited for screening purposes.

The characterization of the SP from *B. adolescentis* revealed that this enzyme exhibits a relative high temperature optimum (58 °C) and a promising thermostability at 60 °C. In contrast, the SP from *L. mesenteroides* has an optimal temperature of only 42 °C and already loses al its activity after 5 min incubation at 60 °C. Keeping in mind that carbohydrate conversions in industry are preferably performed at higher temperatures, *Ba*SP should be a very interesting alternative for *Lm*SP, which has been used for the majority of synthetic applications so far (Kitao *et al.*, 2000; Goedl *et al.*, 2007; Goedl *et al.*, 2008).

Finally, the thermostability of purified SP was found to depend strongly on the enzyme concentration, with higher concentrations being considerably more stable. This effect seems to be less pronounced with crude enzyme preparations. Nevertheless, this phenomenon should not be overlooked during the screening for SP variants with improved thermostability (CHAPTER 3-5).

CHAPTER 3

DETERMINANTS OF THERMOSTABILITY

1. INTRODUCTION

Characterization of the sucrose phosphorylases from *L. mesenteroides* and *B. adolescentis* has revealed that the latter enzyme is considerable more stable than the former (CHAPTER 2). Indeed, *Lm*SP loses nearly all of its activity after only 5 minutes incubation at 60 °C while *Ba*SP is stable for several hours under these conditions. Furthermore, the temperature optimum of *Ba*SP is 16 °C higher than that of *Lm*SP. These intriguing differences will be examined here in more detail. More specifically, the amino acid positions that are responsible for the exceptional thermostability of *Ba*SP will be investigated.

Enzymes have usually been found to be optimally active at temperatures close to that for optimal growth of their source organisms (Vieille & Zeikus, 1996). Clearly, this is not the case for *Ba*SP, as *B. adolescentis* is a mesophilic organism with an optimal growth temperature of 37 °C. To explain this discrepancy, the amino acid composition of its SP will be compared to those typically observed for thermostable enzymes. Furthermore, a sequence alignment of *Ba*SP and *Lm*SP will be generated to reveal amino acid substitutions that could form the basis of their different thermal behavior. The importance of the corresponding positions will be evaluated by means of mutational analysis.

The identification of the determinants of the thermostability of SP enzymes is not only interesting from a scientific perspective but could also be useful for the engineering of the sucrose phosphorylase from *L. mesenteroides*. Although *Ba*SP seems to be the most obvious enzyme for use at high temperature, it displays a different -an perhaps even stricter- acceptor specificity than *Lm*SP (Goedl *et al.*, 2010). If the thermostability of *Lm*SP could be increased by mimicking the sequence of *Ba*SP, a larger range of glycosylation reactions would become available for industrial applications.

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial strains and materials

The constitutive pCXP34h_*Lm*SP expression vector encoding SP from *L. mesenteroides* was described earlier (CHAPTER 2). *E. coli* BL21(DE3) (Novagen) was used as host for recombinant expression and was routinely grown at 37 °C and 200 rpm in LB medium (10 g/L

tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 0.1 g/L ampicillin. All chemicals and medium components were obtained from Sigma or BD Biosciences unless stated otherwise.

2.2 Site-directed mutagenesis

The *Lm*SP gene was mutated by a two-step site-directed mutagenesis procedure using High Fidelity PCR. For the single mutants, the PCR was performed with the corresponding mutagenic primer (Table 3.1) and the expression plasmid pCXshP34_*Lm*SP as template. To create the triple mutant V131L/D139P/E162P, the plasmid of variant D139P was used as template for a PCR reaction with two mutagenic primers that simultaneously anneal to the same strand. The quadruple mutant was constructed in two steps. First the triple mutant G252V/A53D/Y253D was created from the wild-type SP with two mutagenic primers and the PCR product was subsequently used as template to introduce the fourth mutation M84I.

All mutagenic oligonucleotide primers were synthesized by Sigma. PCR cycling conditions were as follows: 95 °C (3 min); 30 cycles of 95 °C (1 min), 55 °C (1 min) and 65 °C (9 min). Each reaction contained 75 ng of plasmid DNA, 2.5 U *Pfu*Ultra DNA polymerase (Stratagene), 10x *Pfu*Ultra HF AD buffer, 0.2 mM of dNTP mix (Westburg) and 0.4 µM of mutagenic primer (when using two primers, 0.4 µM is added of each) in a total volume of 25 µL. After the reaction, the methylated template DNA was degraded by digestion with 10 U of *Dpn*I (New England Biolabs) at 37 °C for 6 h. The PCR mixture was transformed into *E. coli* BL21(DE3) and the transformation mixture was plated on LB medium containing ampicillin. Several colonies were picked and sequenced by LGC Genomics to identify the mutated plasmids. The QIAprep Spin Miniprep kit for plasmid isolation was purchased from Qiagen.

Table 3.1 Mutagenic primers used for site-directed mutagenesis

Primer name	Primer sequence (5'→3')
N19T	TATGCTGATTCGTTGGGCAAAACCTTAAAAGATGTTCATCAAGTC
G36D	GATATTGGAGATGCGATTGATGGGGTTCATTTGTTG
A53D	AGGTGATCGCGGTTTTGATCCAGCCGATTATACTCG
M48I	CTATTTGATGTTTGACTTCATTATTAACCATATTTCTCGTGAATCAG
D109P	AGAAGAATCATGACGATTCAAAGTATAAACCTTTCTTTATTCGTTGGGAAAAGTTC
Q128E	GCGAAAACCGTCCAACAGAAGCCGATGTTGAC
V131L	CCGTCCAACACAAGCCGATCTTGACTTAATTTACAAGC
R137P	TGTTGACTTAATTTACAAGCCTAAAGATAAGGCACCAACGC
D139P	CCGATGTTGACTTAATTTACAAGCGTAAACCTAAGGCACCAACGCAAG
Q144T	GTAAAGATAAGGCACCAACGACCGAAATCACTTTTGATG

N158V	GGCACAACAGAAAACTTGTGGGTGACTTTTGGTGAAGAACAAATTGAC
G161T	GCACAACAGAAAACTTGTGGAATACTTTTACTGAAGAACAAATTGACATTGATG
E162P	CTTGTGGAATACTTTTGGTCCGGAACAAATTGACATTG
G189H	CAACCCTTGAAGACATGGTAAAACATCATGCTAACTTGATTCGTTTG
N208S	TTGCGTATGCAGTTAAAAAAGTTGACACAAGCGACTTCTTCGTTGAGC
G252V	CCCTAAAAAGATCAATGATCATGTTTACTTCACCTATGACTTTGC
M262P	TTCACCTATGACTTTGCATTACCACCGACAACGCTTTACACATTGTATTC
Q275P	GGTAAGACAAATCCGTTGGCAAAGTGGTTGAAGATG
Q287A	GTTGAAGATGTCACCAATGAAGGCGTTCACAACATTGGACACGCATG
T307P	GTGTCGTTGATGCCCGTGATATTCTACCTGATGATGAAATTG
G322T	ACTACGCTTCTGAACAACTTTACAAGGTTACCGCGAATGTCAAAAAG
N335S	ACATATTCATCTGCTTCATACAGCAACCTTGATATTTACCAAATTAAC
G252V/Y253D	AAGATCAATGATCATGTTGATTTCACCTATGACTTTGC

2.3 Enzyme expression and extraction

The wild-type and variant enzymes were produced in 1 L shake flasks and when the cultures reached OD_{600} ~ 0.6, the temperature was lowered from 37 °C to 25 °C. Six hours later, the cells were harvested by centrifugation (7 000 rpm, 4 °C, 20 min) and the obtained pellets were frozen overnight at -20 °C. The enzymes were extracted from the cells by chemo-enzymatic lysis. The frozen *E. coli* pellets were thawed and resuspended in lysis solution (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 50 mM NaCl, 1 g/L lysozyme) supplemented with 0.1 mM phenylmethylsulfonylfluoride. After 30 min incubation at 4 °C, the suspension was sonicated for 2 x 2 min (Branson Sonifier 250, level 3, 50 % duty cycle) and cell debris was removed by centrifugation (15 000 rpm, 4°C, 30 min). The supernatants containing *Lm*SP variants were used for enzyme screening.

2.4 Screening for improved thermostability

The crude enzyme preparations were diluted to approximately 5 U/mL in 100 mM phosphate buffer (pH 7.0). A 100 µL sample of this enzyme solution was heated for 15 min in a water bath at 47 °C, after which the samples were immediately cooled on ice to determine the residual activity. The thermal stability is shown as the ratio of the residual and initial activity, with the ratio for the wild-type enzyme normalized to 100 %. Activity assays were performed continuously at 37 °C with the coupled enzyme assay described in CHAPTER 2. As substrate, 100 mM sucrose in 100 mM phosphate buffer, pH 7.0, was used. One unit (U) of SP activity corresponds to the release of 1 µmole product per minute under these conditions. All thermostability and activity assays were performed in triplicate and had a CV of less than 10 %.

2.5 Inspection of sequence and structure

The primary sequences of SP enzymes were compared by constructing a multiple sequence alignment in Clone Manager Professional Suite (8.0) with the 'Global-Ref' alignment type and the BLOSUM62 scoring matrix. The 3DM database (Bio-Prodict) was used to find correlations between various amino acid positions in SP enzymes. A homology model of *Lm*SP was constructed with the program YASARA using the crystal structure of *Ba*SP (PDB 2gdu) as template. The effect of introduced mutations was also simulated with this program, using AMBER03 as force field for energy minimizations (Wang *et al.*, 2000a).

3. RESULTS AND DISCUSSION

3.1 Selecting targets for mutagenesis

In order to gain more insight in the high thermostability and -activity of *Ba*SP, its primary sequence has been compared with that of *Lm*SP. In the literature, several observations have been reported with respect to the amino acid composition of thermostable enzymes (Vieille & Zeikus, 2001), including:

- *a higher proline and lower glycine content*: introducing prolines and substituting glycines should decrease the entropy of the unfolded state and stabilize a protein. Proline has an exceptional conformational rigidity, while glycine is the smallest and most flexible of the 20 amino acids.
- *a lower asparagine and glutamine content*: substitution of these amino acids can prevent temperature-induced deamidation that affects thermostability of enzymes.
- *a higher content of hydrophobic residues*: hydrophobic effects are one of the major forces for protein folding and should result in a tight packing with as few cavities as possible.
- *a higher content of charged residues*: thermostable enzymes usually contain a more extensive network of ionic interactions at their surface.

As can be seen in Table 3.2, some but not all of these observations apply to SP enzymes. In particular, *Ba*SP does not contain more charged residues than *Lm*SP and more instead of less glycine residues. However, the rules described above are not that strict and different thermostable enzymes typically display different stabilizing features.

Table 3.2 Amino acid composition of *Lm*SP and *Ba*SP

Content (%)	*Lm*SP	*Ba*SP
Hydrophobic residues	28.98	29.17
Charged residues	28.16	26.98
Pro	3.3	4.4
Gly	5.1	6.9
Gln + Asn	9.2	7.0

Charged residues include D, E, K, R, and H while hydrophobic residues include A, I, L, and V.

In a next step, the sequences of *Ba*SP and *Lm*SP have been aligned to identify amino acid substitutions that could be the cause of their different thermostability (Figure 3.1). As both enzymes share a sequence identity of only 35 %, the vast majority of their residues are evidently different. Therefore, we have focused on those substitutions that are well known to influence an enzyme's stability, *i.e.* the introduction of Pro and the removal of Gly as well as Asn and Gln. Many other residues can of course contribute to thermostability of a protein but these are difficult to identify based on sequence analysis alone.

Our sequence analysis revealed 51 positions with crucial differences between *Lm*SP and *Ba*SP (Figure 3.1). To reduce the number of possible mutants to a more manageable size, we have included in our alignment 4 more SP sequences from organisms that are phylogenetically related to either *B. adolescentis* (*i.e. B. longum* and *B. lactis*) or *L. mesenteroides* (*i.e. Lactobacillus acidophilus* and *Streptococcus mutans*). Residues that are highly conserved were then selected as targets for mutagenesis (Table 3.3). One by one, the corresponding substitutions have been introduced in *Lm*SP to evaluate their contribution to thermostability. In addition to these 19 positions, the mutation V131L has also been introduced, as it was previously shown to be advantageous for the thermostability of the related SP from *S. mutans* (Fujii *et al.*, 2006).

Table 3.3 Overview of site-directed mutations introduced in *Lm*SP

Removing Gly	*Introducing Pro*	*Removing Asn/Gln*
G36D	D109P	N19T
G161T	R137P	Q128E
G189H	D139P	Q144T
G252V	E162P	N158V
G322T	M262P	N208S
	Q275P	Q287A
	T307P	N335S

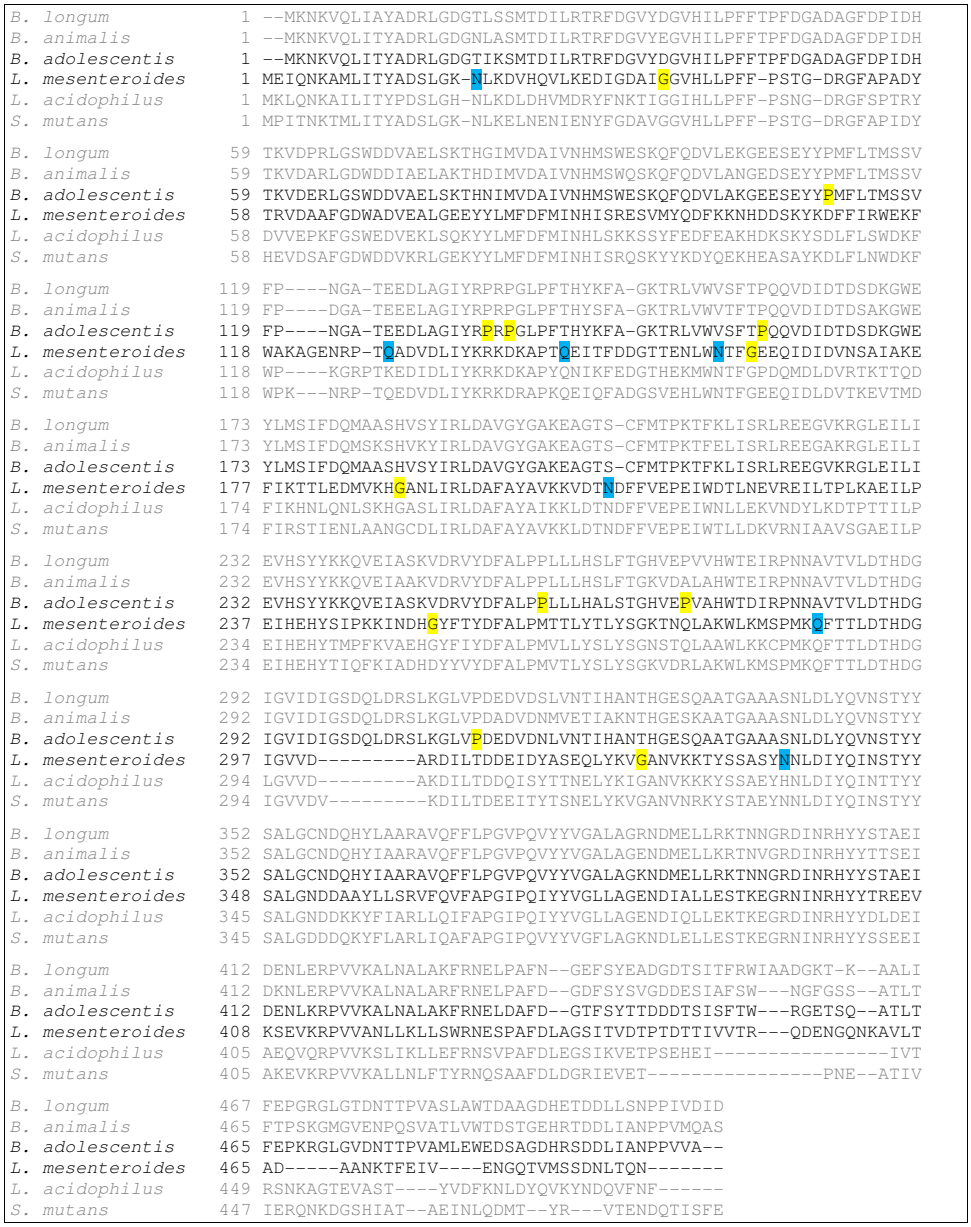

Figure 3.1 Multiple sequence alignment of SP enzymes: X→P & G→X (yellow), N→X & Q→X (blue).

3.2 Screening the *Lm*SP variants

All 20 variants of *Lm*SP were screened for improved thermostability to find out whether the introduced mutations have a significant effect. With the results from CHAPTER 2 in mind, the screening procedure had to be carefully applied. Indeed, the thermostability of SP can vary considerably with the enzyme concentration and this could interfere with our assays, resulting in false positives and/or negatives. Dilutions of unpurified *Lm*SP were found to become increasingly more stable over a large concentration range (Figure 3.2). Remarkably, this effect is exactly the opposite as what was observed with purified SP (CHAPTER 2). An explanation for this discrepancy is not readily available.

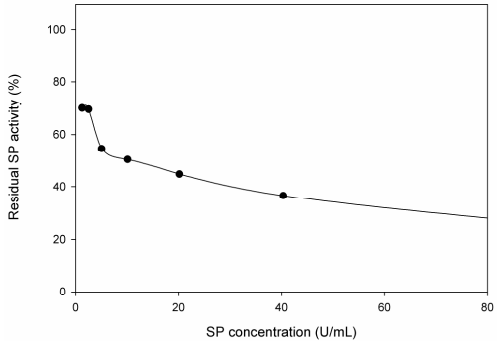

Figure 3.2 Effect of the SP concentration on the thermostability of unpurified *Lm*SP enzyme: 10 min incubation at 47 °C in 100 mM phosphate buffer, pH 6.8 using different *Lm*SP concentrations in the reaction mixture.

To circumvent this problem, the crude enzyme preparations of *Lm*SP variants were all diluted to the same activity of 5 U/mL. The enzyme activity was deemed to be a more appropriate parameter than the protein concentration, as expression levels can differ significantly between variants and the concentration of SP alone cannot easily be determined in a crude preparation. Our strategy of measuring the SP concentration indirectly on the basis of activity relies on the assumption that the specific activity of all variants will be more or less the same. Because none of the mutated amino acids directly interact with the substrate, this assumption is plausible and by far the best way to standardize the thermostability assays.

The thermostability of the enzyme variants was determined by comparing the initial activity with their residual activity after 15 min incubation at 47 °C. Wild-type *Lm*SP was used as reference, which has a residual activity of 31±1.6 %. Only 2 of our 19 variants displayed a significantly

improved thermostability (Figure 3.3). The previously described mutation V131L, resulting in a relative thermostability of 115 %, serves as positive control to prove that our screening procedure is reliable. The other two hits are both realized by the insertion of a proline, namely mutations D139P and E162P, and display relative stabilities of 114 % and 121 %, respectively.

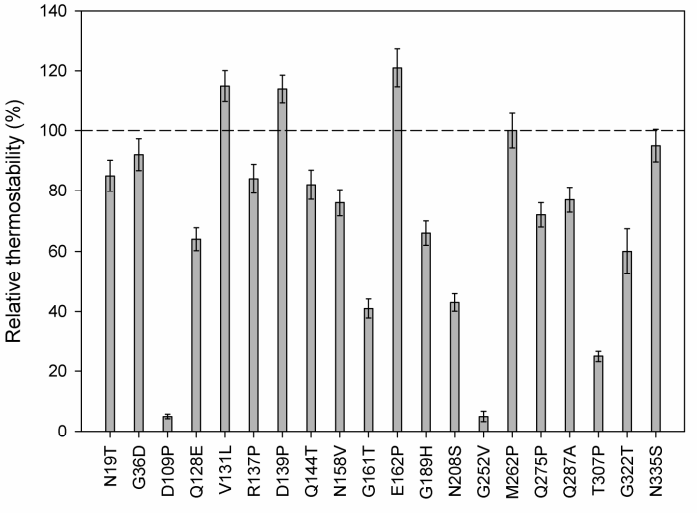

Figure 3.3 Relative thermostability of all *Lm*SP variants after 15 min incubation at 47 °C.
Wild-type *Lm*SP, with a residual activity of 31±1.6 %, is used as reference (100 %).

Very often, the stability of an enzyme can only be efficiently improved by combining multiple mutations that have a rather small effect individually (Matsumura *et al.*, 1986; Yanase *et al.*, 2005). To find out whether our three beneficial mutations have a cumulative effect on the thermostability, a double and triple mutant was constructed and screened (Figure 3.4). The combination of mutations V131L and D139P results in a relative stability of 123 %, which is a significant increase but less than the sum of the individual contributions. The addition of mutation E162P, however, has a much larger effect and increases the relative stability further to 140 %.

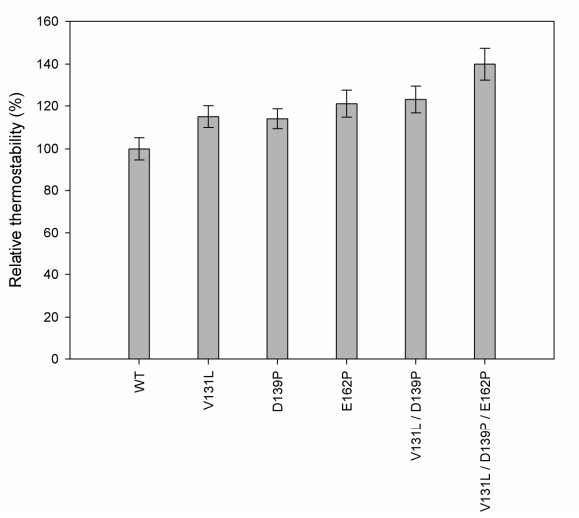

Figure 3.4 Relative thermostability of single, double and triple *Lm*SP mutants after 15 min incubation at 47 °C.
Wild-type *Lm*SP, with a residual activity of 31±1.6 %, is used as reference (100 %).

Remarkably, SP variants D109P and G252V lost more than 90 % of their activity after inactivation. It is, however, possible that the introduced amino acids are only functional within the structural context of the homologous *Ba*SP enzyme. To that end, we checked whether these positions are correlated with other amino acid positions in the 3DM database. This database employs structure-based sequence alignments to reveal evolutionary relationships within a protein family (Kuipers *et al.*, 2009; Kuipers *et al.*, 2010). Every structurally conserved position is designated with a so-called 3D number that is used for the statistical analysis.

The amino acid positions of interest are 109 and 252, of which only the latter is structurally conserved and has a 3D number (130). It correlates strongly with position 84 (3D 70) and to a lesser extent with positions 53 and 253 (3D 40 and 131). Figure 3.5, for example, shows that substituting Gly at position 252 with Val should be accompanied by the mutation M84I. This would even result in the amino acid couple that is preferred by the majority of SP enzymes, and thus can be expected to be optimal for activity and/or stability. Similarly, mutations A53D and Y253D should be introduced in the G252V variant. Unfortunately, the final quadruple mutant showed no activity at all. The additional mutations, however, also show correlations with yet another set of amino acids, forming a large network of correlated positions that vary amongst SP enzymes. Mutating all of them would finally result in a close mimic of the sequence of *Ba*SP but is beyond the scope of this work.

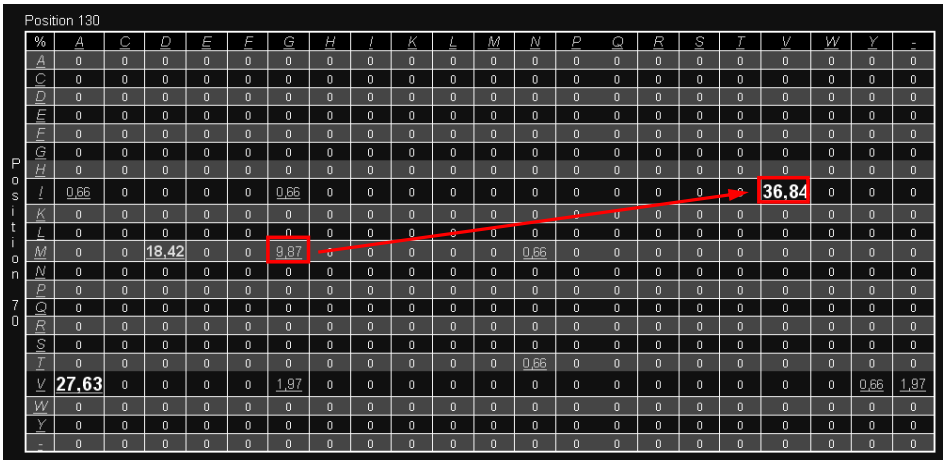

Figure 3.5 The correlation between amino acids at positions 252 (3D 130) and 84 (3D 70). This graph was imported from the 3DM database of family GH-13.

3.3 Location of the beneficial mutations in the 3D structure

In order to obtain a better understanding of the effect of the introduced mutations, their location in the 3D structure was examined. Because the crystal structure of *Lm*SP is not available, a homology model was generated with the program YASARA using the crystal structure of the homologous SP from *B. adolescentis* (PDB 2gdu) as template (Sprogoe *et al.*, 2004; Mirza *et al.*, 2006). All improved variants were found to be mutated in loop regions on the surface of the enzyme. Although the mutated positions are situated in the same region of the structure, they are not close enough to each other to allow a direct interaction. However, long distance effects cannot be ruled out as an explanation for their lack of cumulative behavior.

The influence of the various mutations on their structural environment was also simulated *in silico*. Besides their inherent contribution to stability by reducing the flexibility, additional effects caused by the introduction of prolines were also observed. For instance, a more favorable hydrophobic interaction with I339 could potentially be created by the substitution of D139 (Figure 3.6a). In contrast, substitution of E162 abolishes a salt bridge with K140, the loss of which could potentially be compensated for by creating a hydrophobic interaction with L337 (Figure 3.6b). The previously reported mutation V131L was identified by random mutagenesis and its position in the 3D structure was not described (Fujii *et al.*, 2006). In our model, it can be seen that this residue is involved in a large hydrophobic network, in which leucine might be more favorable than valine (Figure 3.6c).

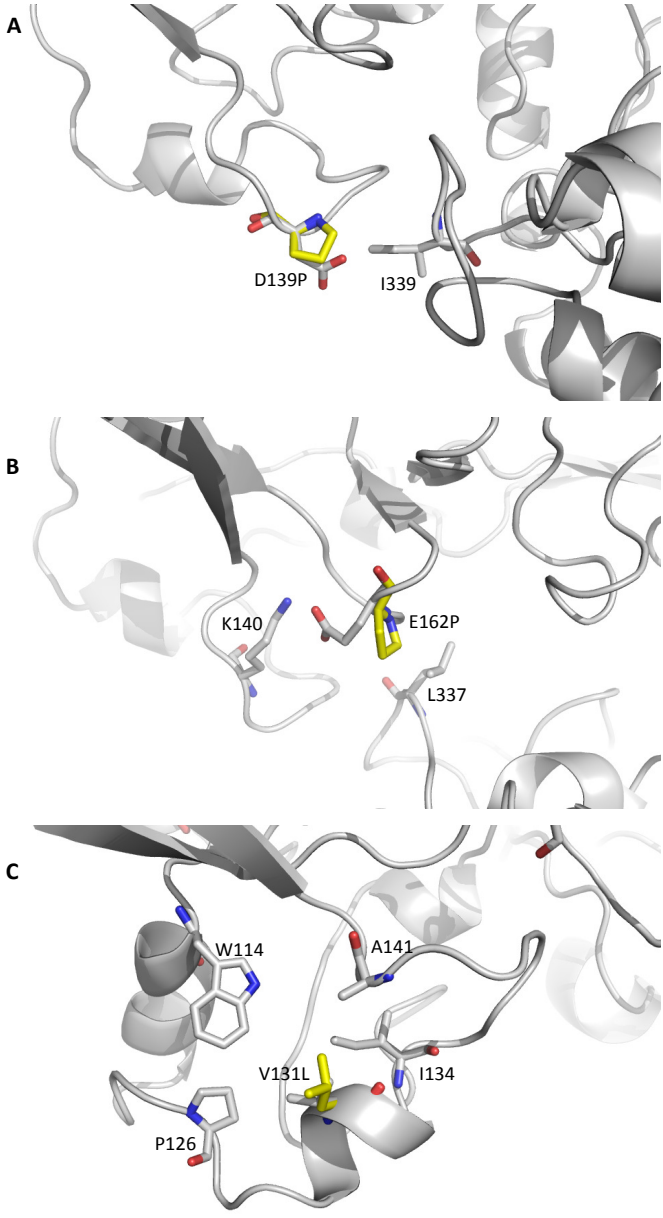

Figure 3.6 Location of the beneficial mutations D139P (A), E162P (B) and V131L (C) in the structure of *Lm*SP. The mutations (in yellow) have been simulated with Yasara and are shown with the wild-type as background (in grey).

4. CONCLUSIONS

In this chapter, the determinants of the exceptional thermostability of *Ba*SP have been examined by mutational analysis. Based on a sequence alignment with *Lm*SP, 19 amino acid substitutions were selected that could have rigidifying effect. Only two of these turned out to contribute to the thermostability of *Ba*SP. Indeed, introducing prolines at positions 139 and 162 of *Lm*SP generates enzyme variants that are 14 and 21 % more stable than the wild-type enzyme. Combining these mutations with the previously identified mutation V131L (Fujii *et al.*, 2006) results in an overall increase of 40 %. In view of the low success rate, further rational engineering of *Lm*SP has been abandoned. As a more stable template is available in the SP from *B. adolescentis*, the latter will be used for future engineering experiments (CHAPTERS 4-5).

CHAPTER 4

DIRECTED EVOLUTION OF THERMOSTABILITY

1. INTRODUCTION

Although the mutagenesis of sucrose phosphorylase from *L. mesenteroides* has generated new insights in the thermostability of the enzyme, none of the obtained variants perform as well as the wild-type SP from *B. adolescentis* (CHAPTER 3). Consequently, we have used the latter as template for further mutagenesis. As rational engineering of thermostability is notoriously difficult, a directed evolution approach will be followed that consists of the randomization of various amino acid positions followed by high throughput screening of the generated libraries.

Several years ago, Reetz and coworkers reported that the thermostability of proteins can be dramatically improved by focusing mutagenesis on the amino acid positions with the highest flexibility (Reetz *et al.*, 2006). These sites are identified on the basis of atomic displacement parameters known as B-factors, which are available from x-ray data and reflect smearing of electron densities with respect to their equilibrium positions. Consequently, in the so-called B-Factor Iterative Test (B-FIT) only those amino acids that display the highest B-factors are subjected to saturation mutagenesis. The best hit obtained at one site is subsequently used as a template for a second round of randomization at another site and the process is repeated iteratively until the desired improvement is achieved. In the case of the lipase from *Bacillus subtilis*, an increase in T_{50} of more than 50 °C could be obtained after five iterations (Reetz *et al.*, 2006).

Libraries can be generated from a single position or by a combination of neighboring residues. The latter allows for synergistic effects between the involved amino acids, but results in much larger libraries that are more difficult to screen. For a 95 % coverage, randomization of a single site requires the screening of 100 colonies (~ 1 microtiter plate), while the simultaneous randomization of two positions requires the screening of 3000 colonies (~ 30 microtiter plates) (Reetz *et al.*, 2008). In any case, a reliable high throughput screening procedure needs to be available to avoid the detection of false positives and/or negatives. More specifically, the CV should be less than 20 % for the complete procedure, including enzyme expression, inactivation and activity measurements.

In this chapter, the application of B-FIT to the SP from *B. adolescentis* will be described. First, the protocols for high throughput screening of thermostability will be optimized with the wild-type enzyme using a robotic platform that is available in the lab. These tools will then be used to process various libraries, whose design will be described in detail.

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial strains and growth conditions

Ultracompetent *E. coli* XL10-Gold cells (Stratagene) were used for expression of mutant libraries and were routinely grown at 37 °C and 200 rpm in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 0.1 g/L ampicillin. The pXSP_*Ba* expression vector encoding SP from *B. adolescentis* was described earlier (CHAPTER 2).

*Dpn*I restriction endonuclease and the QIAprep Spin Miniprep kit for plasmid isolation was obtained from New England Biolabs and Qiagen, respectively. Degenerate primers were synthesized by Sigma. All chemicals and medium components were obtained from Sigma or BD Biosciences unless stated otherwise.

2.2 B-Factor Iterative Test (B-FIT)

The computer program B-FITTER was used to compute the average B-factor of all amino acids in the crystal structure of the SP from *B. adolescentis* (PDB 1r7a). This tool was developed by Reetz and co-workers in 2006 and can be accessed online (Reetz *et al.*, 2006).

2.3 Site-saturation mutagenesis

Site-saturation mutagenesis was performed on the expression plasmid pXSP_*Ba* with the QuikChange Multi Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used for mutagenesis are shown in Table 4.1. PCR cycling conditions were as follows: 95 °C (3 min); 30 cycles of 95 °C (1 min), 55 °C (1 min) and 65 °C (9 min). After the reaction 10 U of *Dpn*I restriction enzyme were added to the reaction mixture and incubated for 6 hours at 37 °C to completely digest parental template DNA. The PCR mixture was transformed into *E. coli* XL10-Gold and the transformation mixture was plate on LB medium containing ampicillin. Several colonies were picked and sequenced by LGC Genomics to check the quality of the libraries.

Table 4.1 Mutagenic primers used for site-saturation mutagenesis

Primer name	Primer sequence (5'→3')
D445_D446	ACGGCACGTTCTCGTACACCACCNNKNNKGACACGTCCATCAGCTTCACCTG
D447	CTCGTACACCACCGATGACNNKACGTCCATCAGCTTCACCT
R304	CTCCGACCAGCTCGACNNKTCGCTCAAGGGTCTCG
R393_K394	CGGCAAGAACGACATGGAGCTGCTGNNKNNKACGAATAACGGCCGCGACATCAATC
L302_D303	ATCGACATCGGCTCCGACCAGNNKNNKCGCTCGCTCAAGGGTCTCGTG
E104	GACGTGCTGGCCAAGGGCNNKGAGTCCGAATACTATCCG

2.4 Optimization of screening procedure of the mutant DNA libraries

2.4.1 Colony-picking, growth and recombinant expression by *E. coli* in MTP's

E. coli XL10-Gold was transformed with pXSP_*Ba* and the transformation mixture was plated on a Q-tray plate (22 cm x 22 cm) using glass beads (6 mm diameter). For optimization of *E. coli* growth in microtiter plates (MTP's), colonies were picked with a QPix2 robotic colony picker (Genetix, UK) and inoculated into 96-well flat-bottomed MTP's (Nunc) containing 175 µL LB + ampicillin per well. The MTP's were incubated overnight (16 h) on a shaker at 37 °C and 250 rpm. These first series of plates are called 'Master plates'. Growth was measured at 595 nm in a microtiter plate reader (Bio-Rad) and the variation was estimated by determining the coefficient of variance (CV = standard deviation / mean x 100 %).

Recombinant enzyme expression was performed in 'Production plates', by replicating overnight grown master plates into new MTP's containing 175 µL LB + ampicillin per well. After incubation for 8 or 16 hours at 37 °C, expression was induced with 0.5 mM IPTG. After 16 or 8 hours of expression at 37 °C, the production plates were centrifuged for 10 min at 3500 rpm in a Rotica 50 RS (Hettich Zentrifugen, USA) to obtain *E. coli* cell pellets. The supernatant could be easily removed from the wells by quickly inverting the MTP's. The MTP's containing cell pellets were frozen at -20 °C until further use.

2.4.2 Enzyme extraction and enzyme activity assays

The following steps were all carried out on a Freedom EVO 200 liquid handling robot (Tecan). Pellets in MTP's were lysed with 300 µL of lysis solution with or without Triton X-100 and lysis was carried out for 10 or 30 min at room temperature (22 °C) or 37 °C. After lysis, the plates were centrifuged at 3500 rpm for 10 min and the supernatants containing SP (crude cell extracts) were used for enzyme screening. SP activity assays were performed with the discontinuous BCA assay measuring the release of fructose as described in CHAPTER 2. Enzyme reactions were

carried out on the robot at 37 °C in MTP's by mixing 10 µL of crude cell extract with 190 µL of pre-heated substrate solution (consisting of 100 mM sucrose in 100 mM phosphate buffer, pH 7.0). After 10 min incubation, 50 µL of sample was taken to determine the amount of released fructose. A 150 µL BCA solution was added to the sample and incubated for 30 min at 70 °C. The absorbance at 540 nm was measured with a microtiter plate reader (Bio-Rad) to determine enzyme activity.

For screening purposes, the optimal lysis conditions were used for extracting SP from *E. coli*. The lysis solution composed of 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 50 mM NaCl and 1 g/L lysozyme, was added to the pellets. After 30 min incubation at room temperature, the plates were centrifuged at 3500 rpm for 10 min and the supernatants containing SP were used for enzyme screening.

2.5 Screening for improved thermostability

Screening libraries for increased thermostability was performed by transferring 40 µL of crude cell extract to a PCR plate (VWR). The plate was sealed with an adhesive PCR film (Abgene) and incubated for 15 min in a water bath at 70 °C. To stop the inactivation, the plates were directly put on ice until further use. Then, the residual SP activity was measured in a MTP by determining the released fructose after 10 min reaction at 37 °C with the discontinuous BCA assay as described earlier. Simultaneously, initial SP activity was also determined with untreated enzyme.

3. RESULTS AND DISCUSSION

3.1 Optimization of the screening procedure

Following transformation of a DNA library in *E. coli*, colonies are formed on a solid medium that -in the ideal case- each contain a different, unique enzyme variant. However, the optimization of the screening procedure was performed with the wild-type SP to evaluate the accuracy (CV) of the experimental protocols (Figure 4.1). *E. coli* XL10-Gold transformed with pXSP_*Ba* was plated on a Q-Tray, and 96 colonies (1 MTP) were picked with an automated colony picker (QPix2). The enzyme induction and extraction, as well as the activity assays and inactivation in MTP-format was performed with a liquid-handling robot (Freedom EVO).

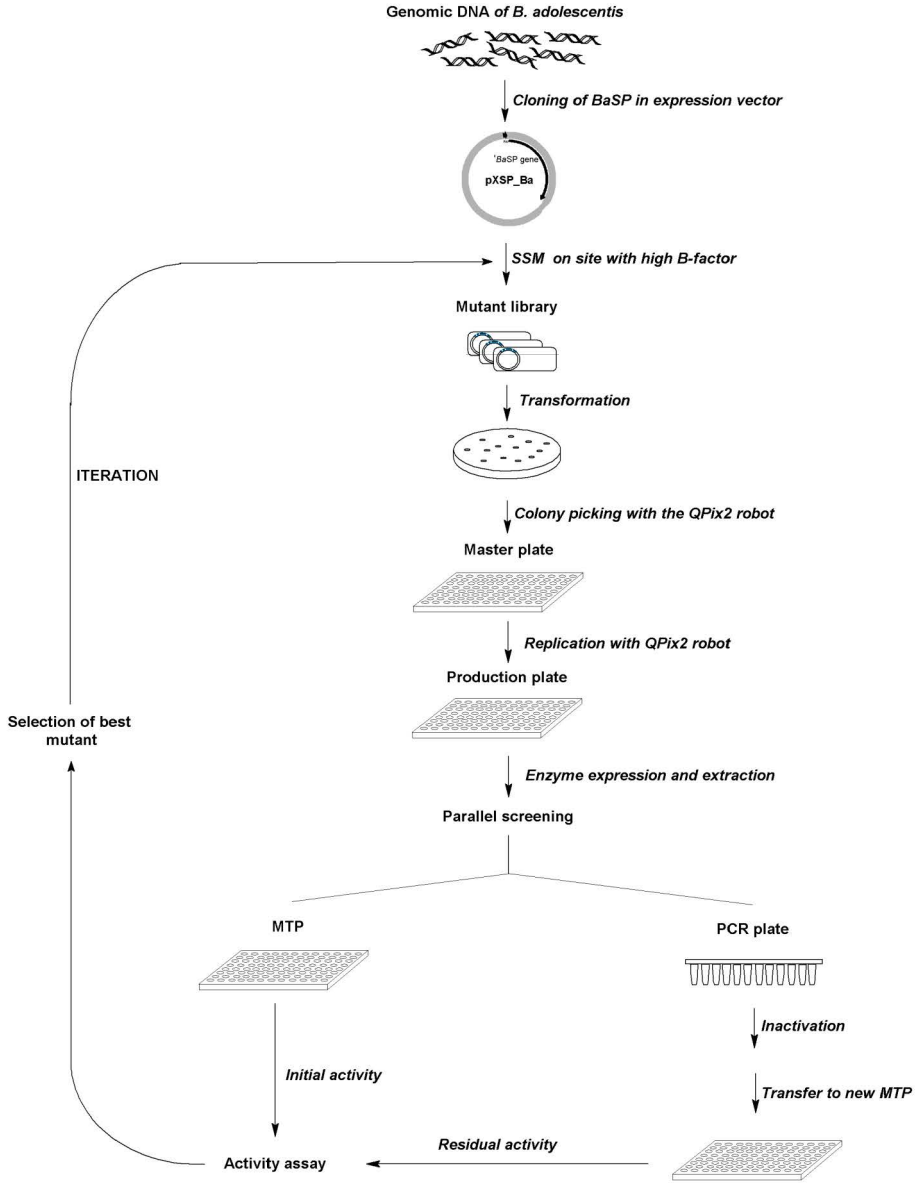

Figure 4.1 Overview of the high throughput screening procedure for thermostability.

3.1.1 Growth and induction in MTP format

Although some dedicated systems have been described for miniaturized growth of micro-organisms, this still is a very challenging task (Duetz, 2007). The different aeration rates in comparison with growth in shake flasks can result in a high well-to-well variation due to position-specific effects. Evaporation of the growth medium from the wells is also an issue during growth in MTP, and cross-contamination should be avoided, especially in directed evolution experiments.

Previous experiments in our laboratory have shown that aeration in MTP's is strongly influenced by both the shaking rate and the culture volume in each well (De Groeve *et al.*, 2010b). A shaking speed of 250 rpm and a culture volume of 175 µL was found to result in optimal growth, with a low variation and a low risk of cross-contamination. Therefore, these conditions were used to grow our master plate. After overnight incubation at 37 °C, relatively high cell densities were obtained (OD_{595} ~ 0.95), with a CV of less than 10 %.

Several production plates were then generated by replicating the master plate with the colony picker. These production plates were used for the optimization of recombinant enzyme expression with the liquid-handling robot. It was found that 6 hours induction at 37 °C after overnight growth yielded the highest and most reliable expression levels.

3.1.2 Enzyme extraction

Triton X-100 is a commonly used detergent for cell disruption and permeabilization. It acts specifically on the cytoplasmic membrane where it binds to the lipid parts. As a consequence, micelles are formed and the cell membrane is disrupted. It was shown that the outer membrane is very resistant to Triton solubilization (Schnaitman, 1971). De Groeve (2009) also demonstrated that Triton X-100 has a rather small effect on cell lysis. A disadvantage of Triton is that it increases the viscosity of the solution, which makes pipetting difficult and increases the variability. Therefore, extracting SP from *E. coli* without Triton X-100 was evaluated. Simultaneously, the optimal incubation time and temperature for the extraction procedure was determined.

Although omitting Triton X-100 from the lysis buffer slightly lowers the activity yield, it significantly improves the accuracy (Table 4.2). The activity was assayed by measuring the concentration of the reducing sugar fructose with the BCA method (A540) after a fixed time period, which will also form the basis of the high throughput screening procedure. Incubating the cells with the lysis buffer for 30 min at room temperature resulted in the highest absorbance with a CV of only 3.2 %.

Table 4.2 Optimization of cell lysis: lysis solution with or without Triton X-100, incubation temperature (°C) and incubation time (min)

	With Triton				Without Triton			
	22 °C		37 °C		22 °C		37 °C	
	10'	30'	10'	30'	10'	30'	10'	30'
Average OD_{540}	1.85	1.86	1.47	1.87	1.64	1.74	1.36	1.72
Stdev	0.19	0.09	0.17	0.09	0.22	0.06	0.19	0.06
CV (%)	10.08	4.94	11.34	4.76	13.12	3.22	13.97	3.60

The activity was determined by measuring the released fructose with the BCA method (A540) after 10 min incubation at 37 °C.

3.1.3 Enzyme inactivation

The simplest procedure for inactivating the cell extracts would consist of incubating a sealed MTP in an oven. Unfortunately, this resulted in a very high degree of variation (CV of more than 35 %), which meant that an alternative solution had to be developed. Floating a MTP in a water bath did not improve the accuracy, probably because the shape of the wells do not allow for an optimal heat flow. Therefore, a PCR plate instead of a MTP was used for inactivation, as these have specifically been developed for incubation at high temperatures and the conic shape of their wells allow for a more intimate contact with the water. Incubating them in a PCR machine would probably have been the best solution but that would have taken up too much time of this costly machinery.

To evaluate the accuracy of the final procedure, 40 µl of the crude enzyme solutions was transferred to a 96-well PCR plate (Abgene). The PCR plate was then sealed and incubated in a warm water bath at 70 °C for 15 min, after which it was immediately cooled on ice. Of the inactivated enzyme solutions, 10 µl was transferred to a new MTP to measure the residual activities. The ratio of the remaining SP activity to the initial SP activity was used as measure for the thermostability of the enzyme. For a plate containing wild-type enzyme in all wells, this resulted in an average residual activity of 28 % with a CV of approximately 15 % (Figure 4.2), which is relatively high but still acceptable. These optimal conditions were used to screen the mutant libraries for improved thermostability.

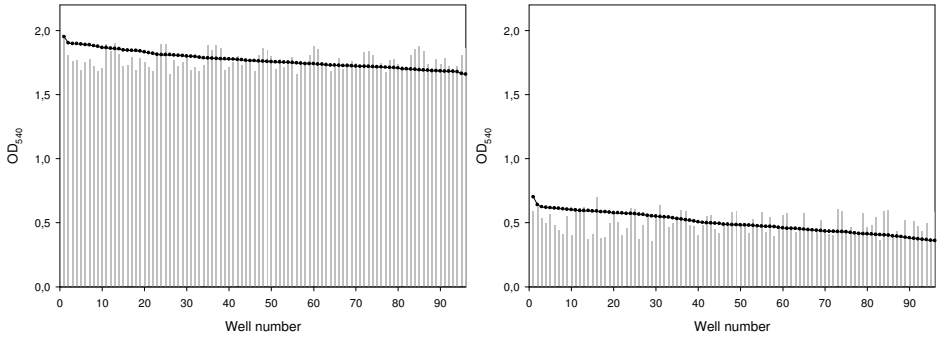

Figure 4.2 SP activity in a MTP before and after 15 min incubation in a water bath at 70 °C. OD_{540} values are plotted per plate position (gray bars) and in descending order (●).

3.2 Design of mutant libraries

The residues with the highest flexibility in the SP from *B. adolescentis* have been identified based on the PDB-file of its crystal structure (Sprogoe *et al.*, 2004). However, this enzyme is composed of two monomers that each display slightly different B-values, resulting in a different ranking for both chains. Therefore, the average B-factor has been used as parameter to select the target residues. Averaging and ranking the B-factors has been performed with the computer program B-FITTER (Reetz *et al.*, 2006). The generated output containing the top 10 positions is shown in Table 4.3. The corresponding residues were chosen as sites for randomization by means of site-saturation mutagenesis. The N- and C- termini have an intrinsically high flexibility and therefore, position A504 will not be addressed.

Table 4.3 Top 10 of residues in the *Ba*SP crystal structure with the highest average B-factor

Rank	Residue	Average B-factor (Å²)	Library
1	D446	42.11	A
2	D445	38.32	A
3	D447	32.89	B
4	R304	30.86	C
5	R393	29.26	D
6	L302	28.44	E
7	D303	27.79	E
8	A504	27.41	-
9	E104	26.48	F
10	K394	26.22	D

All target positions are located in loop regions on the surface of the enzyme, except R393 and K394 that are part of an α-helix (Figure 4.3). From these positions, six libraries were constructed, namely A (D445, D446), B (D447), C (R304), D (R393, K394), E (L302, D303), F (E104). In half of them, two neighboring sites were thus randomized simultaneously to allow for synergistic effects. Saturation mutagenesis was performed using degenerate primers containing the NNK codon, comprising all possible 20 amino acids and one stop codon.

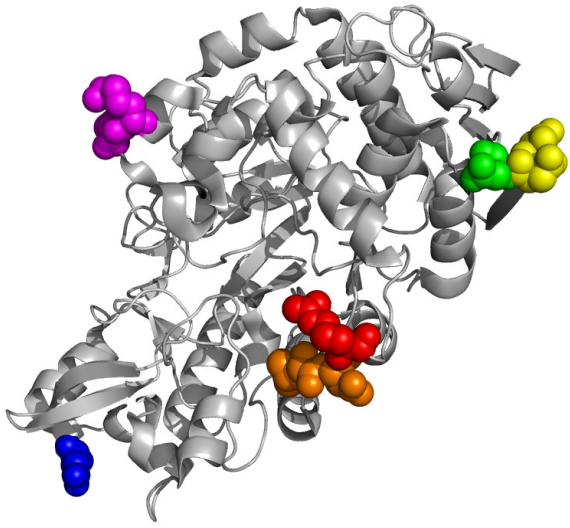

Figure 4.3 The residues in *Ba*SP that were chosen for site-saturation mutagenesis: library A (yellow), B (green), C (red), D (magenta), E (orange) and F (dark blue). Only one monomer is shown.

3.3 Screening for improved thermostability

The libraries described in the previous section were screened for improved thermostability using the protocols optimized with the wild-type SP. More specifically, enzyme inactivation was performed in PCR plates, which were incubated in a water bath at 70 °C for 15 min. The stability of the variants was assessed by comparing their initial activity with the residual activity after inactivation. Under these conditions, the wild-type SP displays a residual activity of 28 ± 4 %.

Libraries B and C were screened first because that required the processing of only a single MTP for each. After calculating the relative thermostabilities, 4 potential hits were identified in the latter and 1 in the former (Figure 4.4). However, the initial activities of these hits were found to be suspiciously low (30-50% of wild-type activity), which could mean that their stability was overestimated due to a low expression level. Indeed, the thermostability of SP is known to be strongly dependent on its concentration (CHAPTER 2). Variations in expression levels for enzyme variants is a common observation and can occur even when just one mutation has been introduced (Tokuriki & Tawfik, 2009b).

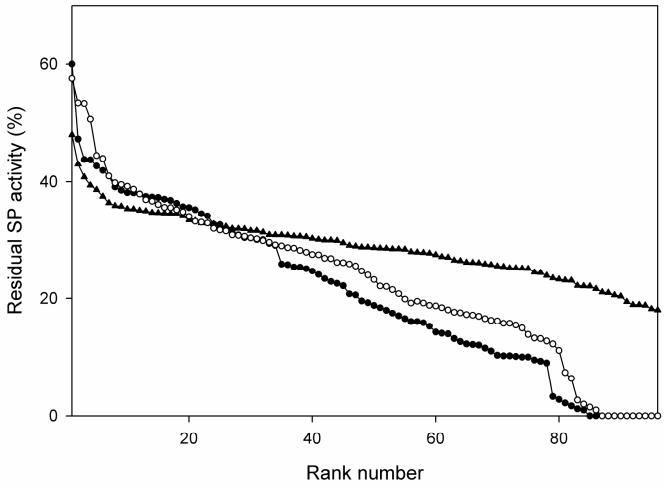

Figure 4.4 Residual activities of a MTP containing wild-type SP (▲) and libraries B (●) and C (○) after 15 min incubation at 70 °C. SP activity was determined at 37 °C with the discontinuous BCA method.

In order to exclude all influence of the expression level, the obtained hits were purified as described in CHAPTER 7, and their thermostability was re-examined after diluting all of them to the same protein concentration. In that case, none of the variants displayed a significantly improved stability compared to the wild-type enzyme. This means that these hits must be considered to be false positives and that randomization at sites B and C actually did not result in increased thermostability. Because of these problems, it will be extremely difficult to accurately perform the high throughput screening of mutant libraries. Therefore, this approach has been complemented with some rational input to facilitate the processing of enzyme variants, as described in the next chapter.

These results do not necessarily mean that it is impossible to improve the thermostability of SP by means of directed evolution. Indeed, a similar approach has successfully been applied to the homologous enzyme from S*treptococcus mutans* (Fujii *et al.*, 2006). Using error-prone PCR, 8 mutations were identified that could be combined to dramatically increase the enzyme's stability. However, the presence of false-positives in that library was not reported and it is not known whether the thermostability of this SP is also influenced by the protein concentration.

4. CONCLUSIONS

In this chapter, protocols have been optimized for the high throughput screening of the thermostability of SP. The inactivation procedure was found to be the most crucial parameter that can result in dramatic variation in residual activity. The best results were obtained by incubating a PCR plate in a water bath, which results in a CV of 15 % for the wild-type enzyme. Using these protocols, two saturation libraries of the SP from *B. adolescentis* have been screened for improved stability. The target residues were chosen based on their high flexibility, as reflected in their B-factors in the enzyme's crystal structure.

Unfortunately, all the hits detected in the mutant libraries turned out to be false positives. In light of the strong dependence of the enzyme's thermostability on its concentration (CHAPTER 2), some problems were to be expected during the high throughput screening. Indeed, variations in expression levels for enzyme variants is a common observation and can occur even when just one mutation has been introduced (Tokuriki & Tawfik, 2009b). In the case of SP, lower expression levels result in a higher thermostability, which explains the presence of false positives. This is, however, not a general feature of proteins and the thermostability of many other enzymes has been improved by high throughput screening of mutant libraries (Eijsink *et al.*, 2005).

A possible solution for this problem would be the co-expression of molecular chaperones to facilitate protein folding. Indeed, chaperones have been shown to level out the concentration of soluble enzyme in mutant libraries by opposing the aggregation of proteins and the formation of inclusion bodies (Wardenga *et al.*, 2008; Tokuriki & Tawfik, 2009a). However, this strategy has not been tried but instead the size of the libraries will be drastically reduced by incorporating additional information, as described in the next chapter. In that way, the generated enzyme variants can be processed individually to counter the effects of protein concentration.

CHAPTER 5

(SEMI)-RATIONAL ENGINEERING OF THERMOSTABILITY

1. INTRODUCTION

The high-throughput screening of SP libraries for improved thermostability has proven to be problematic because of the different expression levels of enzyme variants (CHAPTER 4). Therefore, rational input will be used to limit the size of the libraries to allow the individual processing of enzyme variants. In that way, site-directed mutants can be diluted manually to the same level of activity, effectively eliminating the influence of concentration on the enzyme's stability.

In a first approach, information on amino acid distributions will be extracted from the 3DM database, which contains structure-based sequence alignments of protein families (Kuipers *et al.*, 2010). At each structurally conserved position (referred to by a so-called 3D-number), the frequency of amino acids is calculated, among many other parameters. Based on this information, the randomization in our B-fit libraries can be limited to those residues that are known to be favored by nature. Using a similar approach, the T_m of the esterase from *Pseudomonas fluorescens* was recently increased by as much as 8°C (Jochens *et al.*, 2010). Limited randomization of three amino acid positions generated a 'smart' library that required the screening of only 662 colonies. Furthermore, it was shown that residues that are rarely present at the target positions are detrimental to the enzyme's stability, illustrating the power of natural selection.

In a second approach, mutations will be introduced in a completely rational manner. Although there still are no general rules to stabilize 'any' protein by a limited number of specific mutations, some trends have emerged (Eijsink *et al.*, 2004). Therefore, the 3D structure of *Ba*SP will be examined for amino acid substitutions that might result in additional salt bridges, increased interactions at the dimer interface or stabilization of the dipole of helices. This strategy is by far the most challenging and requires a thorough knowledge of structure-function relationships in proteins.

In this chapter, the (semi-)rational engineering of the sucrose phosphorylase from *B. adolescentis* for improved thermostability will be described. Enzyme variants will be processed individually, and not in the form of a library. Identified hits will be characterized in more detail and the effect of the mutations will be interpreted with respect to their location in the enzyme's crystal structure.

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial strains and materials

E. coli BL21(DE3) (Novagen) was used for expression of *Ba*SP variants and was routinely grown at 37 °C and 200 rpm in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 0.1 g/L ampicillin. The pCXP14h_*Ba*SP expression vector encoding SP from *B. adolescentis* was described in CHAPTER 2.

All mutagenic oligonucleotide primers were synthesized by Sigma. The QIAprep Spin Miniprep kit for isolation of the mutated plasmids was purchased from Qiagen. Mutated plasmids were identified by sequencing (LGC Genomics). All chemicals and medium components were obtained from Sigma or BD Biosciences unless stated otherwise.

2.2 Site-directed mutagenesis

The site-directed *Ba*SP mutants were constructed with the same protocol as for the *Lm*SP variants: a two-step site-directed mutagenesis procedure using High Fidelity PCR and digestion with *Dpn*I (New England Biolabs). For the single mutants, the PCR was performed with the expression plasmid pCXshP14_*Ba*SP as template and the corresponding mutagenic primer (Table 5.1).

Table 5.1 Mutagenic primers used for site-directed mutagenesis

Primer name	Primer sequence (5'→3')
W68D	AACGTCTCGGCAGCGATGACGACGTCGCCGAACTC
S116H	ATGTTCCTCACCATGCATTCCGTGTTCCCGAAC
W171D	CCGATTCCGACAAGGGTGATGAATACCTCATGTC
L306H	CTCGACCGCTCGCATAAGGGTCTCGTGCCGGATG
N325D	AACACCATCCACGCCGATACCCACGGCGAATCC
Q331E	AACACCCACGGCGAATCCGAAGCAGCCACTGGCGCCGCCGCATC
R393N	GACATGGAGCTGCTGAACAAGACGAATAACGGCCGCGAC
N414D	GCGGAAATCGACGAGGATCTCAAGCGTCCGGTCGTC
D445H	TTCTCGTACACCACCCATGACGACACGTCCATCAG
Q460E	CGCGGCGAAACCAGCGAAGCCACGCTGACGTTCGAGCCGAAG
V473H	AAGCGCGGTCTCGGTCATGACAACACTACGCCGGTC
E485H	GCCATGTTGGAATGGCATGATTCCGCGGGAGAC
A498H	TCGGATGATCTGATCCATAATCCGCCTGTCGTC

The double mutant Q460E/E485H was created using the plasmid pCXshP14_*Ba*SP as template for the PCR reaction and two mutagenic primers that simultaneously anneal to the same strand. The double mutant Q331E/R393N was created using the plasmid of SP variant Q331E as template with one mutagenic primer. The triple mutants Q331E/Q460E/E485H and R393N/Q460E/E485H were created using the plasmid of the double mutant Q460E/E485H as template and one mutagenic primer.

2.3 Recombinant enzyme production

Protein expression of the site-directed mutants was performed in 1 L shake flasks using 200 µL LB medium supplemented with 0.1 g/L ampicillin. The recombinant proteins were cultivated at 37 °C for 8 hours after which the cells were harvested by centrifugation (7 000 rpm, 4 °C, 20 min). The obtained pellets were frozen overnight at -20 °C. Crude enzyme solutions were prepared as described in CHAPTER 2 (section 2.3).

2.4 Enzyme purification

The frozen *E. coli* pellets from the site-directed mutants were thawed and disrupted as described in CHAPTER 2 (section 2.7). Cell debris was removed by centrifugation (15 000 rpm, 4 °C, 30 min). The N-terminal His_6-tagged protein was purified by Ni-NTA metal affinity chromatography, as described by the supplier (Qiagen). Buffer Exchange to 100 mM phosphate buffer, pH 7.0, was performed using a centricon in order to remove the imidazole present in the elution buffer. Protein concentrations were measured according to the Lowry-Protein determination (Lowry *et al.*, 1951), with BSA as a standard.

2.5 Determination of enzyme activity

The SP activity of the site-directed mutants was measured at 37 °C using a continuous coupled enzymatic assay in which the release of α-D-glucose-1-phosphate was measured based on the action of PGM and G6P-DH as described earlier (CHAPTER 2). One unit of SP activity corresponds to the release of 1 µmole product per minute from 100 mM sucrose in 100 mM phosphate buffer under the described conditions. All activity assays were performed in triplicate and had a CV of less than 10 %.

2.6 Screening for improved thermostability

In a first screening, the site-directed variants were screened by incubating 100 µl of crude enzyme solution 15 min in a water bath at 70 °C. The inactivation is performed in triplicate in 1.7 mL tubes. Therefore, all cell extracts were diluted with 100 mM phosphate buffer (pH 7.0) to approximately 5 U/mL. After heating, the remaining SP activity was determined. The thermal stability was shown as the ratio of the remaining and the initial activity, with the ratio for the wild-type SP normalized to 100 %.

In a second round, the hits were re-examined by applying heat treatment for one hour in a Gradient thermocycler (Biometra). To that end, 100 µl of purified enzyme solution (diluted to ~7 µg/mL with 100 mM phosphate buffer, pH 7.0) was heated in the range of 58 – 70 °C. After determination of the residual SP activities, the temperature profile was conducted and T_{50}^{60} value was calculated. This latter is the temperature required to reduce the initial enzymatic activity by 50 % within one hour of time.

2.7 Structural interpretation of mutagenesis

The effect of introduced mutations was simulated in the crystal structure of *Ba*SP (PDB 2gdu) with YASARA, using AMBER03 as force field for energy minimizations (Wang *et al.*, 2000a).

3. RESULTS AND DISCUSSION

3.1 Semi-rational design of thermostability

A 3DM alignment of all known SP sequences revealed that only residues D445 (3D 194) and D446 (3D 195) from our B-FIT libraries are structurally conserved. The amino acid distribution at these two positions is shown in Figure 5.1. At the former position, Pro occurs more frequently than Asp, while both Gly and Thr are more abundant at the latter. Consequently, the DD-motif present in *Ba*SP has been substituted with PG, PT, PD, DG and DT by site-directed mutagenesis. Interestingly, a proline residue can also occur at the second position, albeit less frequently and typically in combination with a preceding serine. As Pro is known to be important for protein stability, mutants PP, SP and DP have also been constructed. In total, only eight mutants now have to be screened instead of the 3000 that were needed to cover a saturation library of both positions. The reduction in library size effectively solves the problems caused by differences in

expression levels, as it allows the enzyme variants to be diluted manually to a similar activity level.

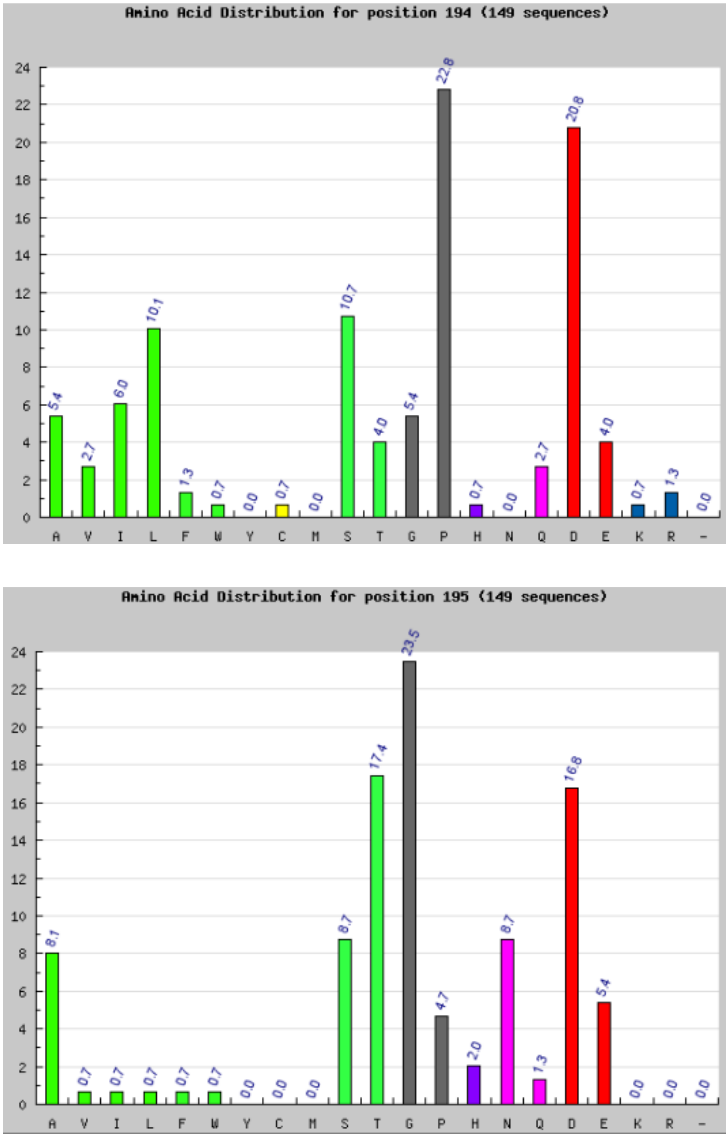

Figure 5.1. Amino acid distribution at positions 445 (3D 194, top) and 446 (3D 195, bottom).

Interestingly, the calculation of root-mean-square deviations (RMSD) based on all PDB files from family GH-113 confirms that residues D445 and D445 are promising targets for mutagenesis (Figure 5.2). Of all structurally conserved amino acids, only the 3D cluster 193 - 197 displays RMSD-values higher than 0.2 with a maximum of 0.36 at 3D 194 (corresponding to D445 in *Ba*SP). It is perhaps not surprising that a correlation can be found between the flexibility of a residue (B-factor) and the deviation in its average position in homologous structures (RMSD). However, the two other positions in this cluster (T444 and T448 in *Ba*SP) were not in the top 10 of highest B-factors and were thus not included in our libraries. As *Ba*SP already contains the consensus residue at both positions (a threonine), these were not given further consideration.

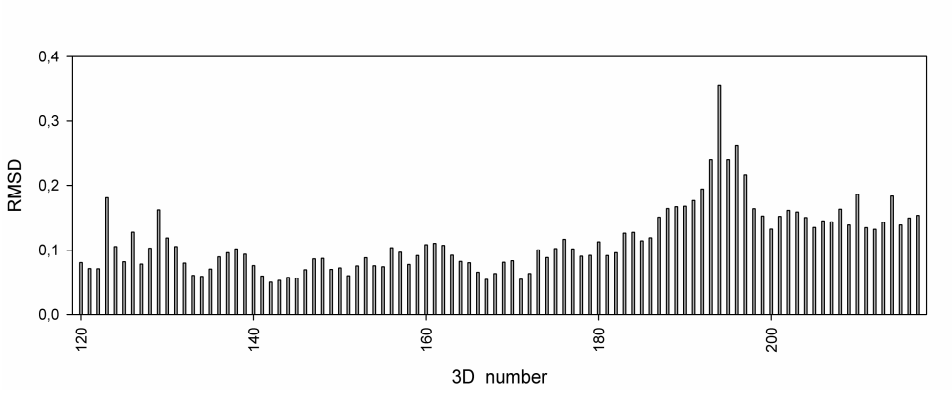

Figure 5.2 RMSD calculations of all PDB files belonging to this 3DM family. Close-up on 3D numbers 120 to 217.

All eight SP variants were tested for improved thermostability by diluting crude enzyme preparations to the same activity level, followed by incubation at 70 °C for 15 min. The enzyme solutions were then immediately cooled on ice and their residual activity was determined. Under these conditions, wild-type SP shows a residual activity of 30 ± 1.7 % for the phosphorolysis of sucrose. The thermostability of the variants is reported relative to that of the wild-type enzyme, which was equaled to 100 % as reference (Figure 5.3).

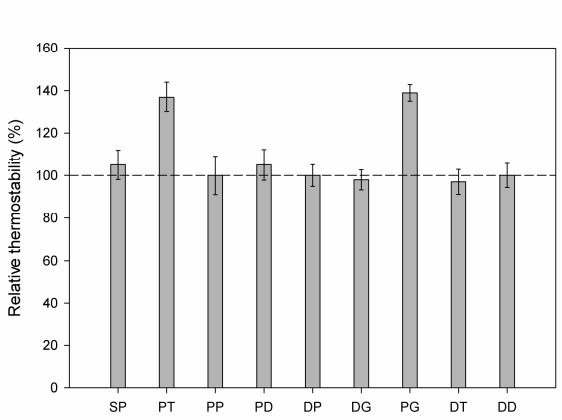

Figure 5.3 Relative thermostability after 15 min incubation at 70 °C of *Ba*SP variants mutated on position 445-446 from DD → XX. Wild-type *Ba*SP, with a residual activity of 30 ± 1.7 %, is used as reference (100 %).

Two *Ba*SP variants showed a significantly improved thermostability after 15 min incubation at 70 °C. Indeed, the substitution of the DD-motif in *Ba*SP with PT and PG resulted in a relative stability of 137 % and 139 %, respectively, compared to the wild-type enzyme. A proline at position 445 thus seems to be crucial for thermostability, albeit not in every combination. In contrast, a proline at position 446 does not seem to have a significant effect. Interestingly, these observations correspond perfectly with the amino acid distributions in the 3DM database (Figure 5.1).

The two hits were then re-examined in more detail after purification by His-tag chromatography. In that way, the samples could be diluted to the same protein concentration instead of the same enzyme activity, excluding any influence from differences in specific activity. In the literature, the so-called T_{50} value is often used to quantitatively describe thermostability, which refers to the temperature required to reduce the initial activity by 50 % within a given period of time (Mansfeld *et al.*, 1997; van den Burg *et al.*, 1999; Zhao & Arnold, 1999; Wang *et al.*, 2000b) After incubation for one hour, wild-type SP from *B. adolescentis* was found to have a T_{50}^{60} of 65 °C, while that of both variants had increased with almost 1 °C (Figure 5.4). These results confirm that introducing a Pro at position 445 has a positive effect on the enzyme's stability.

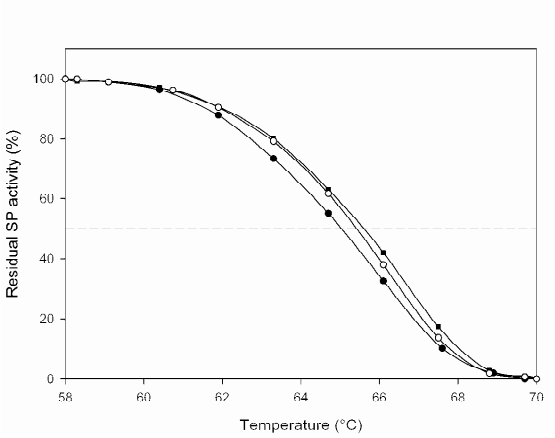

Figure 5.4 Residual activities of wild-type *Ba*SP (●),variant D445P/D446T (■) and variant D445P/D446G (○) after 1 hour incubation at defined temperatures.

3.2 Rational design of thermostability

As a second approach, fully rational-based mutagenesis was applied to the SP from *B. adolescentis* to enhance its stability. From the crystal structure of *Ba*SP (PDB 2gdv), several positions were selected as target for mutagenesis, based on three criteria. First, the complete surface of *Ba*SP was explored for amino acid substitutions that could result in additional electrostatic interactions (Yip *et al.*, 1998). Next, mutations have been introduced at the dimer interface to increase the number of intersubunit interactions, which are known to be important for protein stability (Vieille & Zeikus, 1996). And finally, stabilization of helix dipoles by introducing either negatively charged residues near their N-terminal ends, or positively charged residues near their C-terminal end has also been considered (van den Burg & Eijsink, 2002). Based on these hypotheses, 11 variants of *Ba*SP were created by site-directed mutagenesis (Table 5.1).

Table 5.2 Overview of site-directed mutations in *Ba*SP

	AA mutation
Ion pair network on surface	L306H
	Q331E
	N414D
	D445H
	A498H
	Q460E/E485H
	N325D/V473H
Intersubunit interactions	S116H
	R393N
Helix dipole stabilization	W68D
	W171D

Coincidentally, positions 393 and 445 have again been selected as targets for mutagenesis, while they were also present in our B-fit libraries. This time, the reason is not their high flexibility but because their substitution might result in new, stabilizing interactions. The location of all selected positions in the crystal structure of *Ba*SP is shown in Figure 5.5.

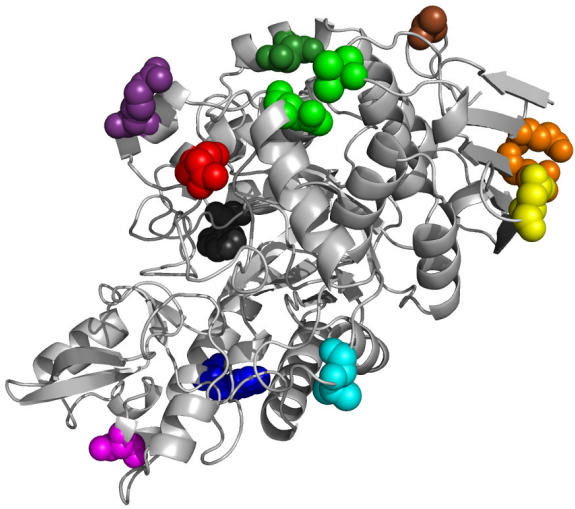

Figure 5.5 The sites in *Ba*SP that were chosen for site-directed mutagenesis. SP variants L306H (cyan), Q331E (red), N414D (dark green), D445H (yellow), A498H (brown), Q460E/E485H, (orange), N325D/V473H (light green), S116H (magenta), R393N (violet), W68D (black) and W171D (blue). Only one monomer is shown.

All SP variants were tested for improved thermostability by diluting crude enzyme preparations to the same activity level, followed by incubation at 70 °C for 15 min. The enzyme solutions were then immediately cooled on ice and their residual activity was determined. Under these conditions, wild-type SP shows a residual activity of 30 ± 1.7 % for the phosphorolysis of sucrose. The thermostability of the variants is reported relative to that of the wild-type enzyme, which was equaled to 100 % as reference (Figure 5.6).

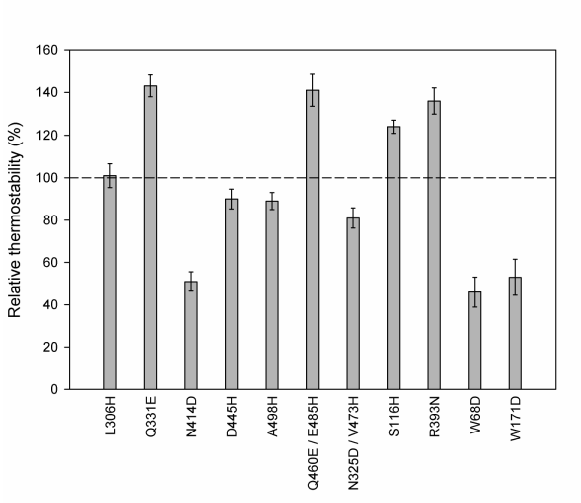

Figure 5.6 Relative thermostability after 15 min incubation at 70 °C of all *Ba*SP variants.
Wild-type *Ba*SP, with a residual activity of 30 ± 1.7 %, is used as reference (100 %).

Four *Ba*SP variants were found to exhibit a significantly improved thermostability. Variants Q331E and Q460E / E485H, both stabilizing SP by introducing new salt bridges, displayed a relative stability of 143 % and 141 %, respectively, compared to the wild-type enzyme. In turn, the additional intersubunit interactions introduced by mutations S116H and R393N result in a relative thermostability of 164 % and 136 %, respectively. Unfortunately, the two variants that were chosen to stabilize helix dipoles, showed a drastically reduced thermostability.

In a next step, the four hits were purified to determine their T_{50}^{60} (Figure 5.7). These values were found to be increased with about 1 °C, for all variants except S116H. Indeed, the temperature profile of the latter enzyme is completely similar to that of the wild-type enzyme, and its identification in the first screening must, therefore, be described as a false positive.

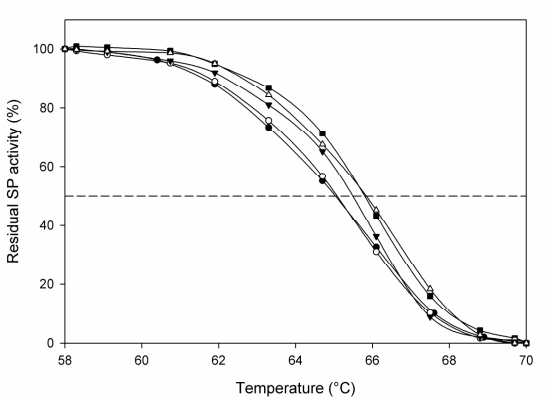

Figure 5.7 Residual activities of wild-type *Ba*SP (●) and variants Q331E (■), Q460E/E485H (Δ), S116H (○) and R393N (▼) after 1 hour incubation at defined temperatures.

Although an increase in T_{50}^{60} with only 1 °C might seem not much, this is a fairly typical result when introducing a single new interaction (Eijsink *et al.*, 2004). Usually, all beneficial mutations have to be pooled in one sequence to achieve much larger improvements. To investigate whether the substitutions identified here can also be combined, the variants Q331E/R393N, Q331E/ Q460E/E485H and R393N/Q460E/E485H were constructed. Their T_{50}^{60} values were found to be about 2 °C higher than that of the wild-type enzyme, meaning that the mutations indeed have an additive effect (Figure 5.8). Furthermore, these variants remain fully active after one hour incubation at 65 °C while the wild-type enzyme looses half of its activity under these conditions.

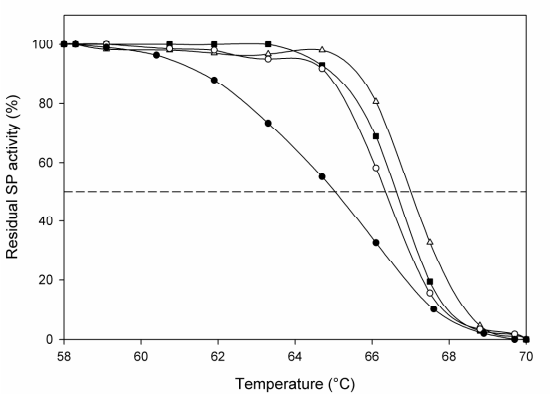

Figure 5.8 Residual activities of wild-type *Ba*SP (●) and variants Q331E/R393N (○), Q331E/Q460E/E485H (■), R393N/Q460E/E485H (Δ) after 1 hour incubation at defined temperatures.

3.3 Structural implications

The structural environment of all beneficial mutations in *Ba*SP has been carefully examined to allow an interpretation of their effect on the enzyme's stability. The substitution of Q331 by a glutamate was performed in order to introduce a salt bridge with H323. This indeed seems very plausible based on the modelled structure of the resulting enzyme variant (Figure 5.9). In turn, residue R393 is located at the enzyme's dimer interface and was believed to induce electrostatic repulsion by interacting with the same residue from the other monomer. Replacing it with an asparagine should alleviate this problem (Figure 5.9).

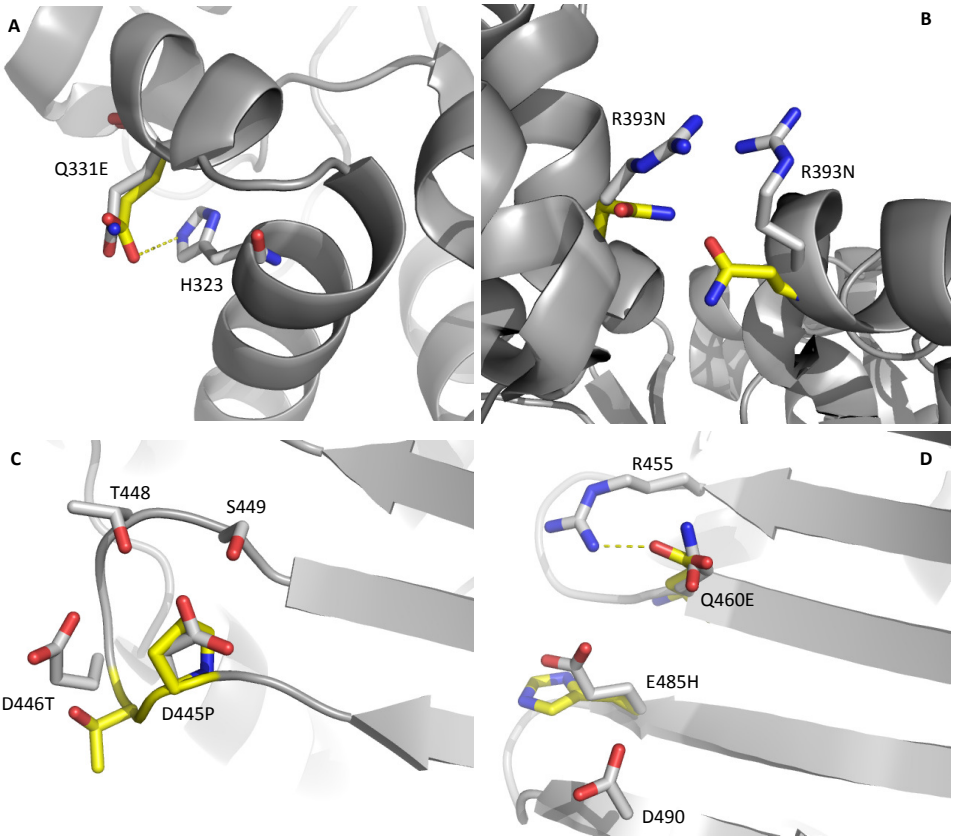

Figure 5.9 Location of the beneficial mutations Q331H (A), R393N (B), D445P/D446T (C) and Q460E/E485H (D) in the structure of *Ba*SP. The mutations (in yellow) have been simulated with Yasara and are shown with the wild-type as background (in grey).

The introduction of a proline residue in variants D445P/D446T and D445P/D446G evidently reduces the flexibility of a loop region at the enzyme's surface. However, by removing both aspartate residues, a network of hydrogen bonds with T448 and S449 seem to have been destroyed, which was not compensated for by introducing a threonine in the former variant (Figure 5.9). Finally, variant Q460E/E485H was constructed for several reasons. The former residue is located close to R455, with which a negatively charged residue should be able to interact more favourably. However, the glutamate at position 485 could then perhaps cause electrostatic repulsion of E460 as well as D490 (Figure 5.9). It has, therefore, been substituted by a histidine to create a network of ion pairs.

4. CONCLUSIONS

In this chapter, semi-rational and fully rational design of thermostability has been applied to the sucrose phosphorylase from *B. adolescentis*. Using input from the 3DM database, the size of our B-FIT libraries could be substantially reduced, allowing the individual processing of enzyme variants. The best hit obtained by this strategy, is the one that contains the amino acids most frequently present in nature. This illustrates the power of natural selection as a tool to guide enzyme engineering experiments. Alternatively, inspection of the protein's crystal structure generated additional targets for mutagenesis. Several variants were obtained with a T_{50}^{60} value that is approximately 1 °C higher than that of the wild-type enzyme. The introduced mutations were found to have an additive effect and combining all of them in a single sequence would probably result in a substantially improved thermostability.

CHAPTER 6

MULTIPOINT COVALENT IMMOBILIZATION

1. INTRODUCTION

The thermostability of an enzyme can be increased in several ways, most notably by means of mutagenesis or immobilization (Unsworth *et al.*, 2007). The mutagenesis of the SP from *B. adolescentis* has already resulted in a number of improved enzyme variants, as described in CHAPTER 5. Their performance is, however, not yet adequate for industrial applications. Therefore, immobilization will be evaluated here as an alternative strategy to enhance the thermostability of SP. An additional advantage of immobilization is that the biocatalyst becomes recyclable for repetitive use or for application in a continuous production process.

Covalent immobilization on an insoluble carrier is known to be a very efficient technique for the rigidification of an enzyme's structure (Mateo *et al.*, 2007). Among the synthetic carriers, Eupergit has long been the industrial standard (Katchalski-Katzir & Kraemer, 2000). More recently, Sepabeads have been shown to display improved properties, such as a low swelling tendency and the availability of various functional groups (Mateo *et al.*, 2007). These supports carry epoxide-groups that react with different nucleophilic residues at a protein's surface, generating a strong multipoint covalent attachment. Physical adsorption of the enzyme is, however, first required and this is mediated by the epoxy-linker. Sepabeads EC-EP contain a short, hydrophobic linker, while that of Sepabeads EC-HFA is longer and more hydrophilic (Figure 6.1). Which one is best for a particular enzyme cannot be predicted but must be examined experimentally.

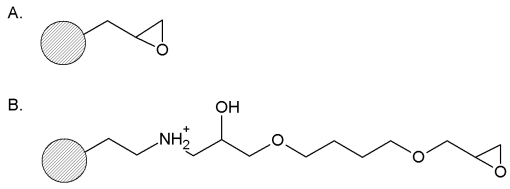

Figure 6.1 Sepabeads EC-EP (A) and EC-HFA (B).

In this chapter, the immobilization of the SP from *B. adolescentis* on EC-EP and EC-HFA will be examined. The immobilization parameters will be optimized by experimental design to maximize the activity yield. The obtained biocatalyst preparation will then be thoroughly characterized to evaluate its performance at high temperatures.

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial strains and materials

The pCXP14h_*Ba*SP expression vector encoding His-tagged SP from *B. adolescentis* was described earlier (CHAPTER 2). *E. coli* XL10-Gold cells (Stratagene) were used for protein expression. Epoxy (EC-EP) and amino-epoxy (EC-HFA) Sepabeads supports were kindly provided by Resindion S.R.L (Mitsubishi Chemical Corporation). All chemicals were analytical grade and purchased from Sigma unless stated otherwise.

2.2 Production and purification of recombinant *Ba*SP

Protein expression and purification of SP from *B. adolescentis* was performed as described in CHAPTER 2. The N-terminal His_6-tagged protein was purified by Ni-NTA metal affinity chromatography and exhibited a specific activity of 161 U/mg. Protein concentrations were measured in triplicate according to the BCA Protein assay (Pierce), with BSA as a standard.

2.3 Enzyme immobilization

The immobilization process was optimized by applying a surface response design. For each condition 0.1 g Sepabeads EC-EP or EC-HFA were added to 5 ml of purified SP solution (0.8 U/mL) and gently stirred at 150 rpm for 48 h and 22 h, respectively. The immobilizates were washed intensively with 100 mM phosphate buffer, pH 7.0. The immobilization yield (Y) and the activity yield (Y_{act}) were defined as followed:

$$Y\ (\%) = \frac{U_{imm}}{U_{free}}$$

$$Y_{act}\ (\%) = \frac{U_{imm}}{(U_{free} - U_{SN} - U_{wash})}$$

With U_{imm} the activity of the immobilized enzyme, U_{free} the activity of the free enzyme added, U_{SN} the remaining activity in the supernatant and U_{wash} the non-covalently bound enzyme in the washing buffer. Immobilization yield at optimal conditions was calculated in triplicate.

A reference suspension was prepared that contains the corresponding amount of inert wet-agarose instead of the support, in all cases the activity of this reference was fully preserved

during immobilization. All statistical analysis were performed with the statistical software environment R (Gentleman & Ihaka, 1997).

At the end of the immobilization process, the immobilized support was treated for 24 h with 5 ml of 3 M glycine solution at pH 8.5 or a 5 % β-mercaptoethanol solution (25 °C), in order to inactivate the remaining epoxy-groups and stabilize the immobilized enzyme (Mateo *et al.*, 2003). After this treatment, the immobilized SP was then washed with an excess of 100 mM phosphate buffer, pH 7.0, to eliminate proteins non-covalently linked to the carrier.

2.4 Determination of enzyme activity

The SP activity was determined by measuring the release of fructose with the bicinchoninic acid (BCA) assay (Waffenschmidt & Jaenicke, 1987). The reactions were analyzed in a discontinuous way, by inactivating samples (5 min, 95 °C) at regular intervals. One unit of SP activity corresponds to the release of 1 µmole product from 100 mM sucrose in 100 mM phosphate buffer at pH 7.0 and 37 °C. All activity assays were performed in triplicate and had a CV of less than 10 %.

The activity of immobilized SP was determined by adding the total amount of washed immobilized enzyme (0.1 g) into 40 ml substrate solution composed of 100 mM sucrose and 100 mM phosphate buffer, pH 7.0. The mixture was incubated in a thermoshaker (Eppendorf) with constant shaking (750 rpm) at 37 °C and samples were analyzed with the BCA assay.

Kinetic parameters for the phosphorolysis direction were determined at optimal temperature and pH by measuring the release of fructose using the BCA method. The concentration of sucrose was varied while phosphate was kept constant at saturating concentration of 100 mM. The kinetic parameters were obtained from non-linear fits of the Michaelis-Menten equation to the initial rates. The protein concentration was measured according to the BCA Protein assay (Pierce), with BSA as a standard.

2.5 Thermal stability assays

Immobilized enzyme was placed in 100 mM phosphate buffer, pH 7.0, and incubated at 60 °C in a Thermoblock (Stuart SBH130D). After 16 h, samples were inactivated and the remaining activity was determined using the BCA method. The influence of the SP concentration on the thermal stability was determined by inactivating different concentration of immobilized enzyme.

3. RESULTS AND DISCUSSION

3.1 Optimization of the immobilization process

Many factors can influence the efficiency of an immobilization process, including the pH and the ionic strength of the immobilization buffer as well as the temperature (Mateo *et al.*, 2007). Here, the effect of these parameters on the immobilization of SP on Sepabeads EC-HFA was investigated by means of experimental design. In the mathematical formulas, these parameters are referred to as x_1, x_2 and x_3, respectively. A phosphate buffer was used in all experiments, and its ionic strength was varied by changing the phosphate concentration.

Table 6.1 2^3 factorial design

Run	x_1	x_2	x_3	Immobilization yield (%)
1	7	0.05	25	57
2	8	0.05	25	56
3	7	0.7	25	4
4	8	0.7	25	37
5	7	0.05	37	56
6	8	0.05	37	55
7	7	0.7	37	11
8	8	0.7	37	37

The parameters x_1, x_2 and x_3 refer to the pH, ionic strength and temperature, respectively.

A screening experiment was performed to determine the relative importance of these factors and their interactions on the immobilization yield. To this end, an experiment was performed according to a full factorial design. The applied levels of the studied factors and the experiment's outcome are listed in Table 6.1. A scree plot revealed that the temperature did not influence the immobilization yield and this parameter was, therefore, set at a fixed value of 25 °C in all consecutive experiments (Figure 6.2). The pH and the phosphate concentration and the interaction term of the pH and the phosphate concentration were selected for further analysis, because these significantly influence the immobilization yield.

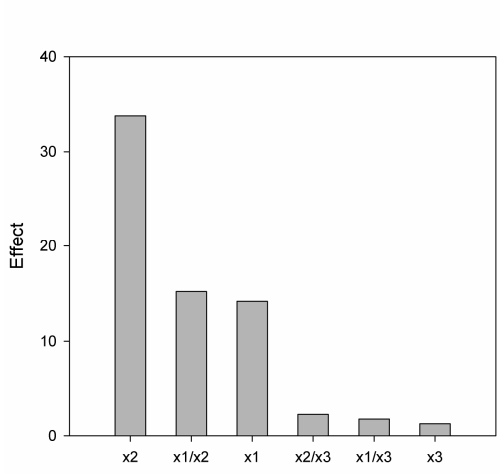

Figure 6.2 Scree plot showing the effect of pH (x_1), phosphate concentration (x_2) and temperature (x_3) on the immobilization of SP on Sepabeads EC-HFA.

The influence of the independent variables pH and phosphate concentration was further evaluated according to a central composite design (CCD), using a generalized linear model. An experiment (CCD 1) was designed around the centre point $(x_1^0, x_2^0) = (7.5,\ 0.05$ M). The variables were appropriately transformed, according to $x_i = (x_i - x_i^0)/\Delta x_i$, with Δx_1 and Δx_2 equal to 0.25 and 0.025 M, respectively. The experiment comprised $(n_c =)$ 4 centre points, 4 factorial points, and 4 axial points. The design and the experiment's outcome are depicted in Table 6.2. The model was accepted because $F \leq F_{n-n_c,n-2,n_c-1,\alpha}$, with α equal to 0.05. The regression model becomes, since the interaction term appeared to be statistically insignificant ($t \leq t_{\alpha/2,n-k}$, with k = the number of parameters):

$$Y = 69.25 - 2.59\ x_1 + 2.56\ x_2 - 1.56\ x_1^2 - 1.81\ x_2^2 + \varepsilon$$

However, the pH optimum did not lie within the investigated experimental domain, hence, subsequent experiments were performed along the path of steepest ascent. The phosphate concentration was kept constant at 0.05 and a step size of 0.2 was used for the pH, in the range of 7.5 - 6.1. Along this path an optimum response was obtained at a pH of 7.1. Hence, a second central composite design (CCD 2) was performed around the centre point $(x_1^0, x_2^0) = (7.1,\ 0.05$ M), and with Δx_1 and Δx_2 equal to 0.2 and 0.025 M, respectively (Table 6.2). Again, the model

was accepted according to Fisher's F-test. Since the interaction term was statistically insignificant, the regression model becomes:

$Y = 69.75 + 2.71\ x_1 - 9.09\ x_2 - 8.51\ x_1^2 - 9.98\ x_2^2 + \varepsilon$

The optimal values were found to be a pH of 7.15 and a phosphate concentration of 0.04 M, resulting in a predicted immobilization yield of 71.93 % (Figure 6.3).

Table 6.2 Central composite designs

Run	x_1	x_2	Immobilization yield (%)	
			CCD 1 $(x_1^0, x_2^0) = (7.5, 0.05\ M)$	CCD 2 $(x_1^0, x_2^0) = (7.1, 0.05\ M)$
1	0	0	66	72
2	0	0	65	68
3	0	0	63	70
4	0	0	67	69
5	-1	-1	60	51
6	-1	1	65	37
7	1	1	62	41
8	1	-1	55	63
9	√2	0	59	58
10	-√2	0	68	54
11	0	-√2	60	66
12	0	√2	66	40

The parameters x_1 and x_2 refer to the pH and ionic strength, respectively, while CCD stands for central composite design.

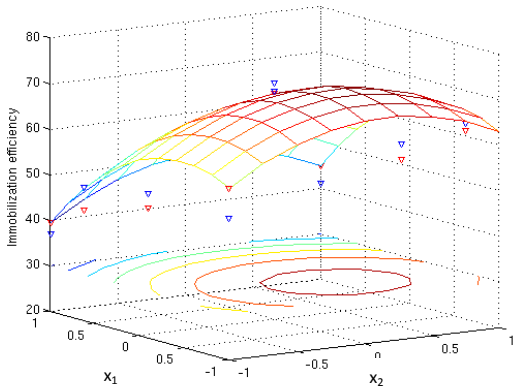

Figure 6.3 Results of the second central composite design.
The parameters x_1 and x_2 refer to pH and ionic strength, respectively.

The same approach was used to optimize the immobilization of SP on Sepabeads EC-EP. In this case, pH, phosphate concentration, temperature and the interaction term of phosphate concentration and temperature significantly influence the immobilization yield (Figure 6.4). The optimal immobilization conditions were a pH of 8.0, a phosphate concentration of 1.5 M and a temperature of 4 °C. The main difference with EC-HFA is the need for high phosphate concentrations. As epoxy groups have a low reactivity towards nucleophiles, physical absorption of the enzyme onto the support is necessary before covalent coupling can take place. In the case of EC-EP, binding of the enzymes is achieved by hydrophobic adsorption, which requires a buffer of high ionic strength. In contrast, EC-HFA can bind enzymes via ionic interactions with the amino functionality present in its linker (Figure 6.1). This difference in binding mechanism is the most likely reason for the variation in immobilization parameters.

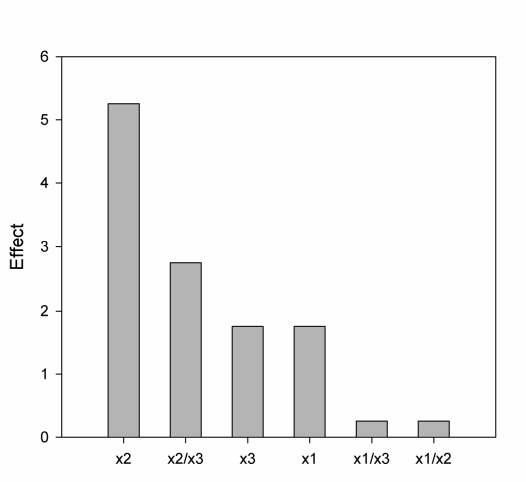

Figure 6.4 Scree plot showing the effect of pH (x_1), phosphate concentration (x_2) and temperature (x_3) on the immobilization of SP on Sepabeads EC-EP.

3.2 Evaluation of the immobilization process

When immobilizing SP on Sepabeads EC-HFA at optimal conditions, total adsorption of the enzyme was achieved within 22 hours (complete loss of activity in the supernatant). At 250 µg protein per gram support (40 U/g support), a recovery of 28 U/g was obtained (yield of 70±2 %). About 10 % of the enzyme was removed during the washing steps and thus non-covalently bound, so it can be assumed that the immobilized enzyme loses approximately 22 % of its activity due to a suboptimal conformation and/or to diffusional problems. The loss of activity during the washing steps could not be avoided by increasing the immobilization time.

Total adsorption of SP on Sepabeads EC-EP at optimal conditions required a longer incubation time, *i.e.* about 48 hours. Furthermore, an immobilization yield of only 15±1 % could be achieved. About 5 % of the enzyme was lost during the washing steps, while the covalently bound enzyme lost 84 % of its activity. Clearly, Sepabeads EC-EP are less suited for the immobilization of SP than Sepabeads EC-HFA.

The presence of 500 mM sucrose during immobilization on Sepabeads EC-HFA and EC-EP did not influence the efficiency of the immobilization. Blocking the free epoxy-groups at the end of the reaction is described in literature to have a stabilizing effect (Mateo *et al.*, 2007).

Unfortunately, this also did not influence the immobilization efficiency. The effect on the thermostability of these two parameters will be discussed in section 3.4.

3.3 Loading capacity of Sepabeads

In order to determine the maximum loading capacity of Sepabeads EC-HFA and EC-EP, different amounts of enzyme (with a specific activity of 161 U/mg) were offered for immobilization. In each case, the activity of the supernatant, the washing buffer and the immobilized biocatalyst was determined (Figure 6.5). When offering 16 mg protein per gram Sepabeads EC-HFA, the maximum loading capacity was all but reached, leading to approximately 530 U/g support. However, this came at a high price: 35 % of bound enzyme was lost during the washing steps, and only 30 % of covalently bounded enzyme was active. Most likely, diffusional problems worsen when higher amounts of enzyme are bound. The maximum loading capacity of Sepabeads EC-EP was not yet reached at 16 mg protein per gram support. Higher loadings could be tested but this is not really efficient because the recovered activity is already lower than for Sepabeads EC-HFA.

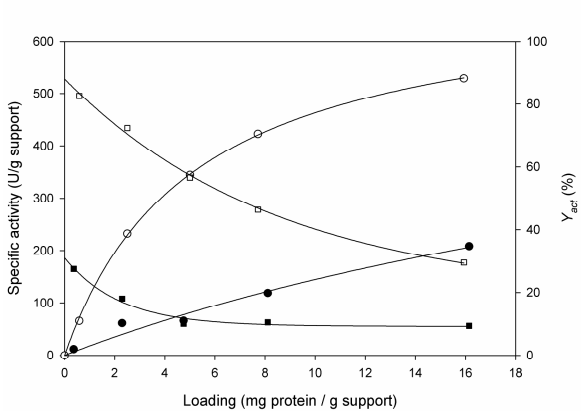

Figure 6.5 Loading capacity of Sepabeads EC-HFA and EC-EP. Enzymatic activity of Sepabeads EC-HFA (○) and EC-EP (●) with different loads of enzyme. Actively bound enzyme of Sepabeads EC-HFA (□) and EC-EP (■).

3.4 Properties of the immobilized enzyme

Many studies have shown that the activity and stability of an immobilized enzyme, can differ from those of its soluble counterpart (Clark, 1994; Mateo *et al.*, 2007). Therefore, the properties

of the SP immobilized on Sepabeads EC-HFA were compared with those of the free enzyme, described in CHAPTER 2. The optimal pH and temperature for activity of the immobilized enzyme were found to be 6.0 and 65 °C, respectively, compared to 6.5 and 58 °C for the free enzyme (Figure 6.6 and Figure 6.7). Furthermore, the immobilized enzyme is active in a broader pH-range, indicating a higher operational stability.

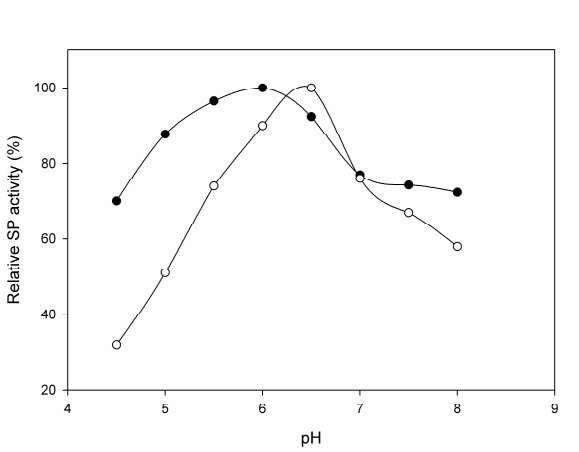

Figure 6.6 The effect of pH on the activity of free (○) and immobilized SP (●) from *B. adolescentis*.
Reactions were performed with 100 mM sucrose in 100 mM phosphate buffer at 37 °C.

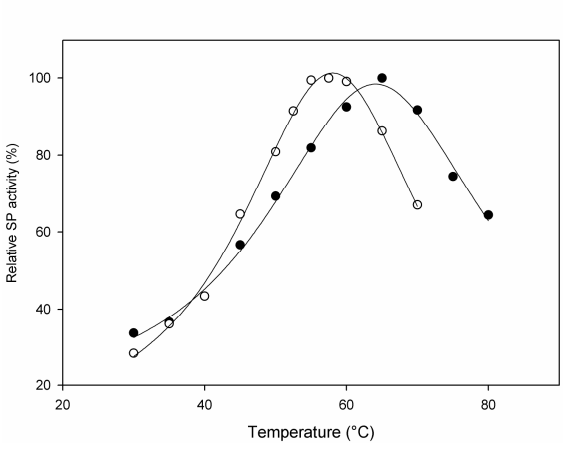

Figure 6.7 Thermoactivity of the free (○) and immobilized (●) SP from *B. adolescentis*.
Reactions were performed with 100 mM sucrose in 100 mM phosphate buffer at pH 7.0.

An evaluation of the influence on thermostability is, however, less straightforward. The immobilized SP retains 65 % of its activity after 16 h incubation at 60 °C, while this varies for the free enzyme according to its concentration. The maximal residual activity that could be reached is 80 %. This, however, requires an enzyme concentration of 40 U/ml, which is not very realistic. In contrast, the stability of the immobilized enzyme is constant in the range of 0 – 800 U/g. Furthermore, its residual activity could be further increased to 75 % by immobilization in the presence of sucrose (500 mM), which does not influence the immobilization yield (Figure 6.8). SP immobilized on Sepabeads EC-EP had a decreased thermal stability: only 30 % of its initial activity was retained after 16 h incubation at 60 °C, whereas the free enzyme retained 70 %. Blocking of the unreacted epoxide-groups by treatment with glycine had no effect on the stability of the immobilized enzyme.

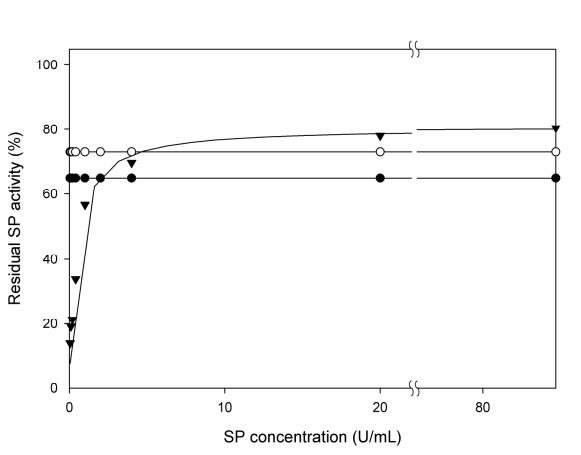

Figure 6.8 Effect of the SP concentration on the thermostability. Residual activity of free (▼) and immobilized enzyme, immobilized in absence (●) or presence of sucrose (○) after 16 h incubation at 60°C.

Finally, the kinetic parameters of the immobilized SP were determined at 65 °C and pH 6.0. The k_{cat} and K_m for sucrose were found to be 310 ± 24 s^{-1} and 9.4 ± 1.3 mM, respectively. The K_m of the immobilized SP is slightly higher than that of the free enzyme (CHAPTER 2), suggesting that immobilization causes diffusional restrictions. The k_{cat} value, in contrast, is higher than that of the free enzyme. Therefore, it can be stated that the loss of activity caused by immobilization is compensated by the higher substrate turn-over that is achieved at the higher optimal temperature (65 °C compared to 58 °C).

3.5 Proof of concept: immobilization of *Lm*SP

In order to determine whether immobilization on EC-HFA is a general strategy to increase the stability of SP enzymes, the procedure was also applied to the SP from *L. mesenteroides*. After incubation of 30 U of purified enzyme with 1 g of Sepabeads EC-HFA for 22 h in 0.04 M phosphate buffer at pH 7.15, a specific activity of 9 U/g was reached (yield of 30 %). The optimal temperature of the immobilized *Lm*SP was 45 °C, which is an increase of 3 °C compared to the free enzyme. This increase is thus less pronounced than that obtained with the SP from *B. adolescentis* (7 °C).

Heating the immobilized *Lm*SP for 5 min at 55 °C resulted in residual activity of 74 %, whereas the free enzyme (0.46 U/mL) shows a residual activity of 50 % (CHAPTER 2). However, it should be taken into consideration that the stability of the free enzyme is dependent on its concentration, while this is not the case for the immobilized SP. Nevertheless, 0.46 U/mL is an industrially relevant concentration and for this concentration the immobilized biocatalyst clearly outperforms the free enzyme.

4. CONCLUSIONS

In this chapter, multipoint covalent immobilization on Sepabeads enzyme carriers (EC) has been evaluated as a means to increase the thermostability of sucrose phosphorylase from *B. adolescentis*. The choice of carrier was found to be of crucial importance, since very good results could be obtained with EC-HFA, while this was not the case with EC-EP. Subsequent optimization of the binding conditions (pH, temperature and ionic strength of the buffer) by means of experimental design resulted in a maximal immobilization yield of 72 %.

The immobilized enzyme has been found to display a number of improved properties. Most notably, the temperature optimum has shifted from 58 °C to 65 °C, and both the thermal and pH stability were considerably increased. Indeed, after overnight incubation at 60 °C, about 65 % of the initial activity was retained. Furthermore, immobilization in the presence of the substrate sucrose further increased this value up to 75 %. The obtained biocatalyst should be useful for application in industrial carbohydrate conversions, providing an opportunity to develop a continuous production process in a packed bed reactor. This will be evaluated in CHAPTER 8.

CHAPTER 7

CROSS-LINKED ENZYME AGGREGATES

1. INTRODUCTION

In the previous chapter, multipoint covalent immobilization was shown to be a very efficient strategy for the stabilization of SP. Indeed, the SP from *B. adolescentis* immobilized on Sepabeads EC-HFA shows a remarkably higher temperature optimum and increased thermal and pH stabilities. Unfortunately, this immobilization technique is quite expensive due to high price of the Sepabeads carrier. Therefore, an alternative immobilization strategy will be evaluated that is based on the cross-linking of enzymes.

Cross-linked enzyme aggregates (CLEAs) have emerged as a novel class of immobilized biocatalysts for use in both aqueous and non-aqueous environments (Cao *et al.*, 2000). Such preparations are obtained by the physical aggregation of the enzymes followed by chemical cross-linking (Figure 7.1). This procedure allows enzymes to be immobilized without the use of a carrier, which not only decreases the cost but also avoids "dilution" of the enzymes' activity (Cao *et al.*, 2003). CLEAs share their beneficial properties with cross-linked enzyme crystals (CLECs) but do not require tedious crystallization procedures (Tischer & Kasche, 1999; Abraham *et al.*, 2004).

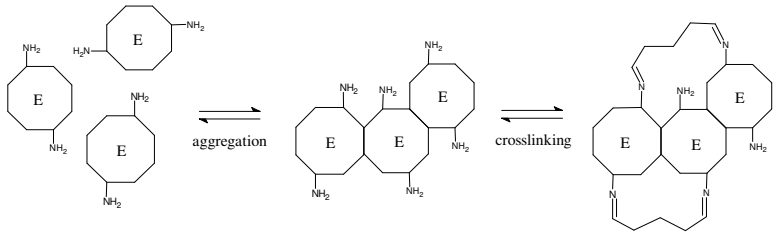

Figure 7.1 General scheme for the production of cross-linked enzyme aggregates. The enzyme molecules are first packed tightly by physical aggregation, after which chemical crosslinking can be performed.

In this chapter, the production of CLEAs of sucrose phosphorylase from *B. adolescentis* will be described to improve its thermal and operational stability.

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial strains and materials

The pCXP14h_*Ba*SP expression vector encoding His-tagged SP from *B. adolescentis* was described earlier (CHAPTER 2). *E. coli* XL10-Gold cells (Stratagene) were used for protein expression. *Tert*-butyl alcohol, glutaraldehyde, and sodium borohydride were purchased from Aldrich-Chemie, Fisher and Acros, respectively. All other reagents were analytical grade and purchased from Sigma.

2.2 Production of recombinant *Ba*SP

SP from *B. adolescentis* was recombinantly expressed in *E. coli* XL10-Gold under control of the constitutive promoter P14. Transformed cells were cultivated in 1 L shake flasks at 37 °C using LB medium supplemented with 0.1 g/L ampicillin. After 8 hours of expression, the cells were harvested by centrifugation (7 000 rpm, 4 °C, 20 min). Crude enzyme solution was prepared by enzymatic lysis of the frozen pellet as described before (CHAPTER 2). The crude enzyme was heat purified by incubation at 60 °C for 60 min. Denaturated proteins were removed by centrifugation (12 000 rpm, 4 °C, 15 min).

2.3 CLEAs production

Aggregates of sucrose phosphorylase were prepared by adding 6 mL of *tert*-butyl alcohol under agitation to 4 mL of heat purified SP enzyme (1.2 mg/mL) at pH 7.0. After 30 min, varying amounts of a 25 % (v/v) glutaraldehyde solution were added to cross-link the enzyme aggregate, and the mixture was kept under stirring for 15, 30, 60 or 120 min. Reduction of the formed imine bond was achieved by adding 10 mL of a solution containing 1 mg/mL sodium borohydride in 100 mM sodium bicarbonate buffer at pH 10.0. After 15 min, another 10 mL was added and allowed to react for 15 min. Finally, the CLEAs were separated by centrifugation (12 000 rpm, 15 min) and washed five times with 100 mM phosphate buffer at pH 7.0. All the steps were performed in a thermoshaker (Eppendorf) at 750 rpm and 4 °C. The immobilization yield is defined as the ratio of the activity detected in the CLEA preparation and that present in the original enzyme solution.

$$Y\ (\%) = \frac{U_{imm}}{U_{free}}$$

2.4 Determination of enzyme activity

The phosphorolytic activity of SP was determined by measuring the release of the reducing sugar fructose from the non-reducing substrate sucrose with the bicinchoninic acid (BCA) method (Waffenschmidt & Jaenicke, 1987). The reactions were analyzed in a discontinuous way, by inactivation of samples (5 min at 95 °C) at regular intervals. One unit (U) of SP activity corresponds to the release of 1 µmole fructose per minute from 100 mM sucrose in 100 mM phosphate buffer at pH 7.0 and 37 °C. To determine phosphatase activity, the samples were also analyzed for the release of glucose (from the α-D-glucose-1-phosphate generated by SP) with the glucose oxidase / peroxidase assay (Werner *et al.*, 1970). One unit of phosphatase activity corresponds to the release of 1 µmole of glucose from 100 mM sucrose in 100 mM phosphate buffer at pH 7.0 and 37 °C. When phosphatase activity was detected, this was subtracted from the values obtained by the BCA method to calculate the net SP activity. All activity assays were performed in triplicate and had a CV of less than 10 %. The protein concentration was measured in triplicate with the BCA Protein Assay kit (Pierce), using BSA as standard.

2.5 Stability assays

To determine the thermostability of SP, soluble or immobilized enzyme was incubated in 100 mM phosphate buffer pH 7.0 in a water bath at 60 °C. At regular intervals, samples were inactivated and the residual activity was analyzed using the BCA method. To evaluate the reusability of SP CLEAs, the biocatalyst was used for several reaction cycles of 1 hour at 60 °C. The enzyme was recuperated by centrifugation (12000 rpm, 15 min) and washed five times with 100 mM phosphate buffer at pH 7.0.

3. RESULTS AND DISCUSSION

3.1 Production and heat-purification of SP

The SP gene from *B. adolescentis* LMG 10502 was recombinantly expressed in *E. coli* XL10-Gold. After chemo-enzymatic cell lysis, a crude enzyme preparation was obtained with a specific SP activity of approximately 13 U/mg at 37 °C. As the SP is more stable than most endogenous *E. coli* proteins, the enzyme could be partially purified by means of heat treatment (Table 7.1). In this way, all phosphatase activity was removed, which would otherwise degrade the α-D-glucose-1-phosphate (αG1P) produced by SP.

Table 7.1 Heat purification of SP from *B. adolescentis*

Incubation at 60 °C (min)	SP activity (U/mL)	Phosphatase activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)
0	35.1	2.7	2.6	13.5
20	35.2	0.7	1.6	22.0
40	35.0	0.0	1.5	23.3
60	35.1	0.0	1.2	29.3
90	35.0	0.0	1.2	29.2

Assays were performed at 37 °C using 100 mM sucrose in 100 mM phosphate buffer pH 7.0 as substrate. SP activity corresponds to the release of fructose, while phosphatase activity corresponds to the release of glucose (from the αG1P formed by SP).

After one hour incubation at 60 °C, the specific activity increased to 29 U/mg and the concentration of soluble protein dropped from 2.6 to 1.2 mg/mL. The latter was completely due to the loss of contaminating proteins, as no decrease in SP activity was observed under these conditions. Longer incubation times did not result in a further increase in specific activity. Although the final enzyme preparation was estimated to be only 20 % pure, as judged by SDS-PAGE (not shown), a higher level of purity is not required for most applications and would probably not be economical at an industrial scale.

3.2 Production of SP CLEAs

The first step in the preparation of CLEAs consists of the aggregation of the enzymes, which can be achieved by the addition of salts, organic solvents or non-ionic polymers (Cao *et al.*, 2000). The choice of the additive is important, because it can result in enzymes with slightly different three-dimensional structures. Ammonium sulfate is the most widely used precipitant for protein purification, but gave unsatisfactory results with sucrose phosphorylase. High concentrations of the salt were required (~ 70 % w/v) to aggregate this enzyme and generated a gelatinous suspension that was difficult to centrifuge. Precipitation was, therefore, performed with *tert*-butanol instead. A solvent concentration of 60 % (v/v) resulted in complete removal of SP activity from the supernatant after centrifugation. The precipitate was redissolved in phosphate buffer without loss of activity, indicating that the aggregation procedure did not damage the structural integrity of the protein.

In the second step, the aggregated enzyme molecules were chemically cross-linked to obtain an immobilised biocatalyst. Glutaraldehyde (GA) is generally used for that purpose, as it contains two aldehyde groups that can form imine bonds with lysine residues from two enzyme molecules

(Figure 7.1). It is well known that the immobilization yield strongly depends on the incubation time of the cross-linking step as well as on the GA / protein ratio (Wilson *et al.*, 2009). These parameters have, therefore, been optimized for the production of CLEAs of SPase (Figure 7.2).

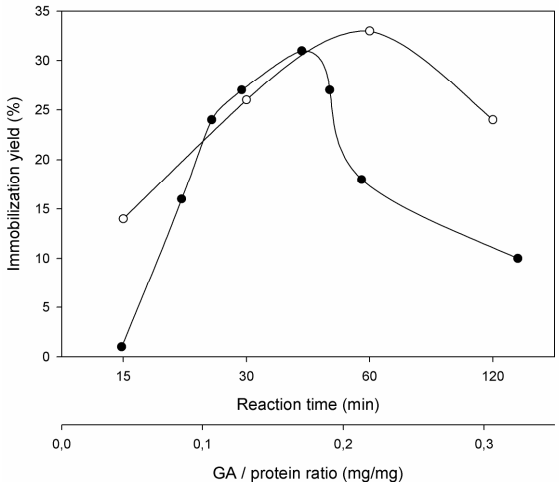

Figure 7.2 The effect of the cross-linking ratio (●) and reaction time (○) on the immobilization yield of SP from *B. adolescentis*. The immobilization yield is defined as the ratio of the activity detected in the CLEA preparation and that present in the original enzyme solution.

A maximal immobilization yield of 31±2 % was achieved at a GA / protein ratio of 0.17 mg/mg and an incubation time of one hour. Higher ratios and longer incubation times resulted in a considerable reduction in catalytic activity, most likely because glutaraldehyde then started to react with residues in the active site. Indeed, this problem can sometimes be avoided by the use of polymeric cross-linkers that are too large to access the active site (Mateo *et al.*, 2004), but these were not tested in this work. Even under optimal conditions, the activity recovered in the CLEA preparation was lower than that of the free SP, which means that diffusion limitations were operative and/or that the catalytic site had undergone some kind of distortion.

3.3 Characterisation of SP CLEAs

Many studies have shown that the activity and stability of an immobilized enzyme can differ considerably from that of its soluble counterpart (Clark, 1994; Mateo *et al.*, 2007). Therefore, the

properties of the CLEAs were compared with those of the native SP. The optimal pH and temperature for phosphorolytic activity of the immobilized enzyme were found to be 6.0 and 75 °C, compared to 6.5 and 58 °C, respectively, for the soluble enzyme (Figure 7.3 and Figure 7.4). Cross-linking thus results in an enzyme whose temperature optimum has increased by an impressive 17 °C. Furthermore, the immobilized enzyme was active in a broader pH-range, indicating a higher operational stability.

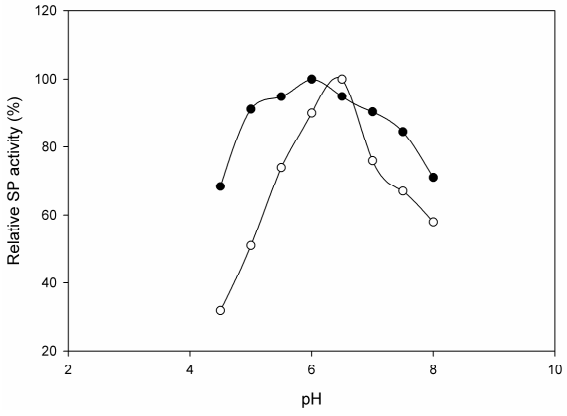

Figure 7.3 The effect of pH on the activity of soluble (○) and immobilized (●) SP from *B. adolescentis*.
Reactions were performed with 100 mM sucrose in a 100 mM phosphate buffer at 37 °C.

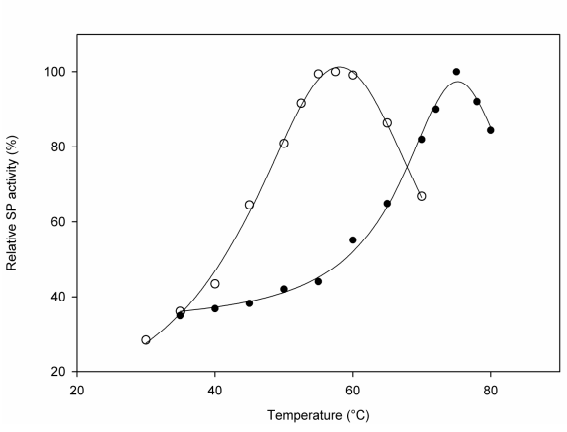

Figure 7.4 The effect of temperature on the activity of soluble (○) and immobilized (●) SP from *B. adolescentis*.
Reactions were performed with 100 mM sucrose in a 100 mM phosphate buffer at pH 7.0.

To determine the thermostability of the SP preparation, the enzyme was incubated at 60 °C and its residual activity was measured at several points in time. The CLEAs were found to retain full activity after one week incubation, whereas the free enzyme lost 20 % of its activity after only 16 hours incubation. The stability of the biocatalyst was, therefore, dramatically improved by the cross-linking process. As industrial carbohydrate conversions are preferably performed at 60 °C, the properties of these CLEAs will undoubtedly allow the development of novel processes of high economic value.

One of the major advantages of immobilization is that it leads to an enzyme preparation that can be recycled, which often is a key determinant of its industrial potential. CLEAs can be easily recycled by either filtration or centrifugation (Cao *et al.*, 2003), and the latter strategy has been used in our experiments. Centrifugation at high speeds (12000 rpm) was found to be required for the precipitation of CLEAs and completely remove phosphorolytic activity from the supernatant. To evaluate the mechanical stability of the biocatalyst under these conditions, several reaction cycles of one hour at 60 °C were performed with thorough washing in between. After ten cycles, no loss of activity was detected, revealing the excellent operational stability of the new enzyme preparation.

4. CONCLUSION

In this chapter, the preparation of cross-linked enzyme aggregates has been evaluated as a means to increase the thermostability of the sucrose phosphorylase from *B. adolescentis*. To minimize the costs, the enzyme was simply purified by heat treatment, which removed all contaminating phosphatase activity. Subsequent optimization of the cross-linking step resulted in an immobilization yield of 31 %. The obtained CLEAs remain fully active after one week incubation at 60 °C and can be recycled at least ten times for the repeated conversion of sucrose into α-D-glucose-1-phosphate. Furthermore, the optimal temperature of activity was found to increase by as much as 17 °C, while the pH profile has significantly broadened. This low-cost and easy procedure should allow the development of industrial processes to exploit the different glycosylation reactions catalyzed by sucrose phosphorylase.

CHAPTER 8

PRODUCTION OF GLUCOSE-1-PHOSPHATE

1. INTRODUCTION

Several preparations of the sucrose phosphorylase from *B. adolescentis* have been developed that display exceptional thermostability. Indeed, the enzyme remains fully active for several hours at 60 °C when immobilized on Sepabeads EC-HFA (CHAPTER 6), while its cross-linked enzyme aggregate (CLEA) is even stable for more than one week (CHAPTER 7). The performance of these biocatalysts under process conditions will now be evaluated for the production of α-D-glucose-1-phosphate (αG1P).

Glycosyl phosphates are key intermediates in the synthesis of nucleotide sugars, the activated donors of glycosyl transferases (Bulter & Elling, 1999). They can be produced by chemical synthesis, but these procedures suffer from low yields and lack of anomeric selectivity (Posternak, 1950). Biocatalytic synthesis typically relies on kinases, but the need for ATP as phosphate donor is a major limitation for their application in large-scale processes (Zhao & van der Donk, 2003). Glycoside phosphorylases are, therefore, a very attractive solution, as they only require cheap inorganic phosphate for the production of a glycosyl phosphate from a di- or oligosaccharide (Goedl *et al.*, 2007).

Whole-cell bioconversions often are a very practical and cost-effective alternative for the use of isolated enzymes in industrial applications (Wandrey *et al.*, 2000). The cell wall can, however, form a serious barrier for the diffusion of substrate and product, unless specific transport mechanisms are available. Fortunately, this obstacle can be overcome by partial permeabilization of the cell membrane with various solubilizers (Felix, 1982). This process has to be carefully optimized to avoid loss of enzyme by leakage through the damaged membranes. In that way, enzymes can be confined to their stable cellular environment while their access to the substrate is no longer limited.

In this chapter, the production of α-D-glucose-1-phosphate by the enzymatic conversion of sucrose at 60 °C will be described. To date, such a procedure has not yet been reported in the literature. A biocatalytic process with immobilized SP as well as a whole-cell bioconversion with a recombinant *E. coli* will be developed. Furthermore, the equilibrium constant of the reaction will be determined to evaluate the theoretical maximum product yield, and a simple strategy for the purification of αG1P will be presented.

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial strains and materials

The pCXP34h_*Lm*SP and pCXP14h_*Ba*SP expression vectors encoding His-tagged SP from *L. mesenteroides* and *B. adolescentis*, respectively, were described earlier (CHAPTER 2). *E. coli* XL10-Gold cells (Stratagene) were used for protein expression, while *E. coli* BL21(DE3)RIL cells (Stratagene) were used for permeabilization experiments. Amino-epoxy (EC-HFA) Sepabeads were kindly provided by Resindion S.R.L (Mitsubishi Chemical Corporation). All reagents were analytical grade and purchased from Sigma, unless stated otherwise.

2.2 Production of recombinant SP

SP from *B. adolescentis* was recombinantly expressed in *E. coli* XL10-Gold under control of the constitutive promoter P14. Transformed cells were cultivated in 1 L shake flasks at 37 °C using LB medium supplemented with 0.1 g/L ampicillin. After 8 hours of expression, the cells were harvested by centrifugation (7 000 rpm, 4 °C, 20 min). Crude enzyme solution was prepared by enzymatic lysis of the frozen pellet as described in CHAPTER 2. His-tagged purified and heat purified SP was prepared as described in CHAPTERS 2 and 7, respectively.

2.3 Immobilization of SP

The multipoint covalent attachment of SP on Sepabeads EC-HFA was performed in triplicate with unpurified, partially purified or His-tagged purified enzyme at optimal conditions, as described in CHAPTER 6. Cross-linked enzyme aggregates of SP were prepared with heat purified enzyme solutions at optimal conditions, as described in CHAPTER 7.

2.4 Permeabilization

2.4.1 Growth conditions and storage of cell cultures

SP from *L. mesenteroides* was recombinantly expressed in *E. coli* BL21(DE3)RIL under control of the constitutive promoter P34. Protein expression was performed in shake flasks at 37 °C using LB medium supplemented with 0.1 g/L ampicillin. Once the cultures reached the exponential phase (OD_{600} ~ 0.6), the recombinant proteins were further expressed at 25 °C. After

overnight incubation, 1 mL samples were stored in four different ways in order to retain as much SP as possible (Table 8.1).

Table 8.1 Different storage conditions

	Temperature (°C)	Additives
Pellet[a]	-20	-
Pellet[a]	4	-
Cell culture	-20	250 µl glycerol
Washed pellet[b]	4	1mL 100 mM phosphate buffer, pH 7.0 + 250 µl glycerol

[a] 1 mL cell culture was centrifuged for 2 min at 10 000 rpm; [b] Pellet of 1 mL cell culture was washed with 100 mM phosphate buffer, pH 7.0 and centrifuged for 2 min at 10 000 rpm.

After 6 days incubation at the respective condition, the harvested cells were washed with 100 mM phosphate buffer, pH 7.0 and disrupted by enzymatic lysis using the EasyLyse™ Bacterial Protein Extraction Solution (Epicentre Biotechnologies). After overnight incubation in the freezer, the thawed cells were resuspended in 200 µL of EasyLyse solution and after a 5 min incubation at room temperature, the lysis mixture was centrifuged at 18 000 rpm. The supernatant (cell extract) was tested for SP activity with the BCA method.

An overnight incubation at -20 °C of a pellet obtained from 1 mL cell culture was used as reference. After enzymatic lysis using EasyLyse, the cell extract showed a SP activity of 2.25 U/mL.

2.4.2 Optimization of the permeabilization procedure

Samples of 1 mL frozen cell cultures, supplemented with 250 µL glycerol (optimal storage conditions), were thawed and centrifuged at 10 000 rpm for 2 min. The harvested cells were permeabilized by treatment with toluene and Triton X-100. The concentration of the permeabilizers was 1, 5 and 10 (v/v) %, and 0.5, 1, 2, 5 and 10 (v/v) %, respectively, both diluted in 100 mM phosphate buffer, pH 7.0. The cells were suspended in the respective pre-cooled permeabilization buffer and incubated on ice for different times. Then, cells were centrifuged for 2 min at 10 000 rpm and resuspended in 100 mM phosphate buffer, pH 7.0. SP activity of this suspension (SU) was determined using the BCA method. This suspension was then kept at 4 °C or room temperature until further use. To test cell leakage, samples were taken at different times and centrifuged for 2 min at 10 000 rpm. The supernatant (SN), which is the extracellular solution of the permeabilized cells was tested for SP activity using the BCA method. After cell treatment, the permeabilization buffer (PB), separated from the cells, was also tested for SP activity with the BCA method.

At the start of each experiment, a non-permeabilized sample was taken as reference. Therefore, a frozen sample was thawed, centrifuged and the harvested cells were disrupted by enzymatic lysis using the EasyLyse solution. The SP activity of this cell extract was measured with the BCA assay. The SP activity of the permeabilized cells was then compared with this reference value (= maximal SP activity).

2.5 Determination of enzyme activity

The release of the reducing sugar fructose from the non-reducing substrate sucrose was measured with the BCA method (Waffenschmidt & Jaenicke, 1987). One unit (U) of SP activity corresponds to the release of 1 µmole fructose from 100 mM sucrose in 100 mM phosphate buffer at pH 7.0 and 37 °C.

The release of αG1P was assayed in a coupled enzymatic system with PGM and G6P-DH (Silverstein *et al.*, 1967; Weinhausel *et al.*, 1997). The assay was performed in a discontinuous way as described in CHAPTER 2.

The activity of immobilized SP on Sepabeads EC-HFA was determined by adding the total amount of washed immobilized enzyme (0.1 g) into 40 ml substrate solution composed of 100 mM sucrose and 100 mM phosphate buffer, pH 7.0. The mixture was incubated in a thermoshaker (Eppendorf) with constant shaking (750 rpm) at 37 °C and samples were analyzed in triplicate with the BCA assay.

The protein concentration was measured in triplicate with the BCA Protein Assay kit (Pierce), using BSA as standard.

2.6 Reaction equilibrium and conversion of sucrose

The K_{eq} for the phosphorolysis of sucrose was determined in 200 mM phosphate buffer, pH 7.0 at 37 °C and 60 °C. The assays contained equimolar concentrations of sucrose and phosphate, and 2 U/mL purified SP. Equilibrium concentrations of reactants and products were determined on a Varian ProStar HPLC using an aminex HPX-87H column (Bio-Rad) at 30 °C. The eluent consisted of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The K_{eq} was calculated using Eq. (1).

$$K_{eq} = \frac{[\alpha G1P][D-fructose]}{[sucrose][phosphate]} \quad (1)$$

2.7 Continuous production of αG1P

Heat purified *Ba*SP, covalently attached to 20 mL Sepabeads EC-HFA at optimal immobilization conditions, was packed into a column (diameter of 1.4 cm, 20 mL, packing height of 13.5 cm) equipped with a water mantle at 60 °C. The column was equilibrated with 100 mM phosphate buffer, pH 7.0. The substrate solution contained 500 mM sucrose in 500 mM phosphate buffer, pH 7.0, and was brought to reaction temperature (60 °C) through incubation in a water bath. The solution was pumped through the packed bed at a constant flow rate of 0.75 mL/min. At certain times, 1 mL samples were taken and analyzed on a Varian ProStar HPLC using an aminex HPX-87H column (Bio-Rad) at 30 °C as described above.

2.8 Purification of αG1P

After the production of αG1P with immobilized *Ba*SP as described in the previous section, the product was purified by removal of sucrose, fructose and glucose by treatment with baker's yeast (20 g/L). This reaction took place at 30 °C for 8 hours. At certain times, 1 mL samples were taken and analyzed on Varian Prostar HPLC using an aminex HPX-87H column (Bio-Rad) at 30 °C as described above. Phosphate was precipitated by the addition of magnesium acetate and ammonia (Ashby *et al.*, 1955). Biomass and precipitated phosphate were removed by filtration through a Seitz EKS filter. The pH of the filtrate was brought to pH 7.0 and the next step was to concentrate this filtrate in a rotary evaporator (Büchi, Rotavapor R-134) at 60 °C until a brix of 45 was obtained (evaporative crystallization). Then, the solution was slowly cooled to 0 °C to grow the crystals, which were subsequently filtered through Whatman paper, washed with ethanol and dried overnight at 70 °C.

3. RESULTS AND DISCUSSION

3.1 Reaction equilibrium of sucrose phosphorolysis

To know the maximal yield of α-D-glucose-1-phosphate (αG1P) that can be obtained by the phosphorolysis of sucrose, the K_{eq} was determined experimentally at pH 7 and either 37 °C or 60 °C. Starting from 200 mM of both substrates, the reaction was allowed to proceed until equilibrium was reached. The final concentrations of reactants and products corresponded to a K_{eq} value of 5.73±0.55 and 5.63±0.41 at 37 °C and 60 °C, respectively. Therefore, it can be concluded that temperature has little effect on the K_{eq} for the phosphorolysis of sucrose. However, these values are considerably different from the K_{eq} of 44 (at 30 °C and pH 7)

determined in an earlier report (Goedl *et al.*, 2007). The latter value indicates that a yield of 87 % can be reached, while our experiments generate a yield of only 71 %.

3.2 Continuous production with immobilized SP

In CHAPTER 6, the immobilization of purified sucrose phosphorylase on Sepabeads EC-HFA was described. This resulted in a highly stable and reusable biocatalyst with a temperature optimum of 65 °C. For industrial applications, however, the purification of an enzyme by chromatographic techniques, e.g. His-tag purification, is rather expensive. Therefore, the immobilization of crude SP preparations will first be evaluated.

Of a crude enzyme preparation with a specific activity of approximately 13 U/mg, 4 U were added to 0.1 g Sepabeads EC-HFA and immobilized at optimal conditions. After 22 hours, an immobilization yield of 17 % was obtained. When immobilizing 4 U of purified SP, however, a yield of 70 % was achieved (Figure 8.1a). This indicates that there is no preferential binding of SP on Sepabeads. On the contrary, some of the other proteins present in the crude preparation seem to have a higher affinity for the resin. As the contaminating phosphatases might also become stabilized upon immobilization, a simple purification step by means of heat treatment was performed. As described in CHAPTER 7, all phosphatase activity can be removed in that way. When immobilizing 4 U of heat purified SP on 0.1 g Sepabeads EC-HFA at optimal conditions, an immobilization yield of 50 % was achieved, which is a significant increase compared to the 17 % for immobilization with unpurified SP (Figure 8.1).

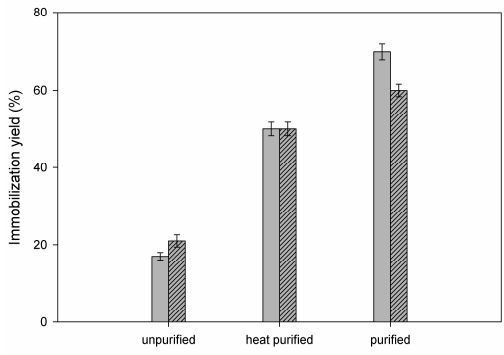

Figure 8.1 Immobilization yield of unpurified, heat purified and His-tagged purified SP on Sepabeads EC-HFA when loading 40 U/g support (solid) or 1.4 mg/g support (shadowed).

However, the total protein concentration in 4 U of unpurified, heat purified or His-tag purified SP is different, resulting in different protein/resin ratios. As described in CHAPTER 6, a higher protein/resin ratio results in a lower immobilization yield. Therefore, an immobilization experiment was performed with the same protein/resin ratio for all three SP preparations. In that case, an immobilization yield of 21, 50 and 60 % was obtained for unpurified, heat purified and His-tag purified enzyme, respectively (Figure 8.1). Heat purified SP thus presents an interesting solution that is free from contaminating phosphatase activity and generates a reasonable immobilization yield.

The immobilized biocatalyst was then used for the continuous production of αG1P in a packed bed reactor. The reaction was carried out at 60 °C and with a constant flow rate of 0.75 mL/min, corresponding to an average residence time of 24 min. The degree of conversion for sucrose (400 mM) was 69 %, corresponding to a productivity of 179.5 g/L/h. Note that the conversion rate remained constant up to extended reaction times of 240 h, emphasizing the excellent stability of immobilized SP under the operational conditions. These results also revealed the major influence of sucrose on the stability of this biocatalyst. In absence of sucrose, the immobilized enzyme lost already 25 % of its activity after 16 h incubation at 60 °C, as described in CHAPTER 6.

This is the first time that a production process with SP is described at elevated temperatures, Enzymatic synthesis of αG1P has been studied previously using immobilized native SP from *L. mesenteroides* but all these reported processes were carried out at 30°C in contrast with 60°C reported here (Guibert & Monsan, 1988; Pimentel & Ferreira, 1991; Soetaert *et al.*, 1995). In conclusion, recombinant SP from *B. adolescentis* immobilized on Sepabeads EC-HFA seems to be an interesting novel biocatalyst for the production of αG1P.

3.3 Repetitive batch production with CLEAs of SP

As described in CHAPTER 7, cross-linked enzyme aggregates of SP display an exceptional thermostability, retaining all of their activity after 1 week incubation at 60 °C. To evaluate the efficiency of the SP CLEAs in a production process, the phosphorolysis of sucrose into αG1P was monitored at 60 °C and pH 7.0 until maximal conversion. The reaction was performed with 500 U of CLEAs in a solution containing 1 M of both sucrose and inorganic phosphate. After about 20 h, the reaction was finished and 0.7 M αG1P was produced. This corresponds perfectly with the yield of 71 % obtained with the free enzyme (section 3.1). In view of the exceptional mechanical stability of the CLEAs during recycling (CHAPTER 7), it should be possible to repeat this reaction at least seven times in one week time. In that way, more than 1 kg of αG1P would be produced with only about 50 mg of protein, which still would be fully active.

3.4 Production by whole-cell bioconversion

The cell wall and cellular membrane are essential components of microbial cells. They confer rigidity and provide semi-permeable barriers to chemical species that come in contact with the cell. Ideally, for a whole-cell bioconversion, the starting material should be transported into the cell without impedance so that the rate of the production is dictated only by the metabolic functions in the cell. Once the products are synthesized inside the cells and accumulated to an appropriate level, releasing products from cellular confinement and allowing products to be collected outside the cell without breaking the cell comprise the ideal scenario that would allow cells to be utilized more than once or continuously (Chen, 2007). To achieve this target, it is sometimes necessary to partially or totally permeabilize the cell.

The permeability of the membrane is determined by its composition. Numerous studies have documented the dependence of membrane composition of *E. coli* on various environmental growth conditions (McGarrity & Armstrong, 1975; Arneborg *et al.*, 1993; Shokri *et al.*, 2002). These include growth rate, medium composition, growth phase, temperature and stress conditions. Thus, by varying the growth conditions, the permeability of the membrane can be changed. However, this simple strategy has not been widely exploited. In this study, preliminary tests were performed on samples from different growth phases and from different *E. coli* strains. The best results were obtained with *E. coli* BL21(DE3)RIL grown until the late-stationary phase. Recombinant SP from *L. mesenteroides* was chosen as biocatalyst because the temperature optimum of this enzyme corresponds well with that of the host organism.

3.4.1 Storage of cultivated cells

Before we optimized the permeabilization procedure, long-term stability of SP within the cultivated cells was evaluated. To that end, cells were harvested and stored for 6 days at different conditions as described in the Materials and methods section (Table 8.1). The cells were disrupted by enzymatic lysis and the obtained extracts were measured for SP activity and compared with the reference value of 2.25 U/mL, which corresponds to the activity extracted from fresh cells (Figure 8.2).

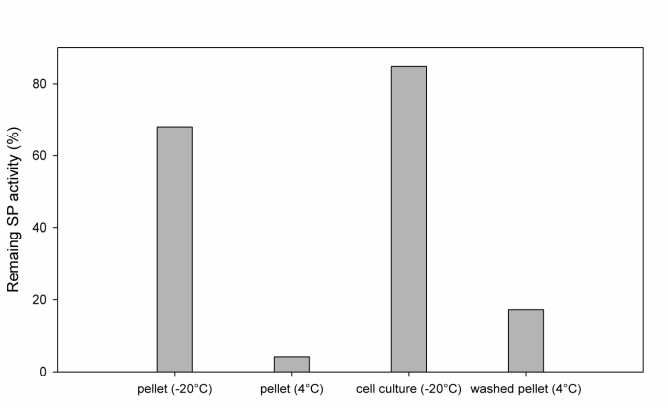

Figure 8.2 Remaining SP activities after different storage conditions.
A fresh extract with an activity of 2.25 U/mL is used as reference (= 100 %).

Figure 8.2 shows that freezing of cultivated cells in the presence of glycerol was the best method for long-term storage: 85 % of the initial SP activity could be retained after 6 days. Subsequent experiments also revealed that no cell leakage was observed, not even after weeks of storage at -20 °C. When the cells were stored at 4 °C, the enzyme was not very stable resulting in a residual activity of less than 20 % after 6 days. Therefore, for all successive experiments, the cultivated cells were stored at -20 °C in the presence of glycerol.

If we want to use and recycle permeabilized cells for the production of αG1P, it is important that not only sucrose can enter the cells but also that the produced αG1P can leave the cells. Preliminary experiments showed that similar activities could be measured with the discontinuous BCA method (release of the reducing sugar fructose) and the coupled enzymatic assay (release of αG1P). This means that both products can leave the treated cells in equimolar amounts and therefore, the simple and cheap BCA method was used to determine the SP activity of treated cells in the next section.

3.4.2 Permeabilization procedure

Untreated cells showed almost no measurable SP activity. Therefore, two different permeabilizing agents were evaluated as a means to increase the diffusion of substrate through the cell membrane, namely toluene and Triton X-100. The former is an organic solvent while the latter is a non-ionic surfactant, both of which disturb the integrity of the outer membrane (Felix, 1982; Vaara, 1992).

The SP activities of the recombinant *E. coli* cells (SU) treated for 5 min with different concentration of toluene and Triton X-100 are shown in Figure 8.3 and Figure 8.4, respectively. After 30 min incubation at 4 °C, the extracellular solution of the permeabilized cells (SN) was tested for activity, to evaluate whether enzyme had leaked out off the cells. The activities were then compared with the crude SP preparation, which had an SP activity of 2.25 U/mL.

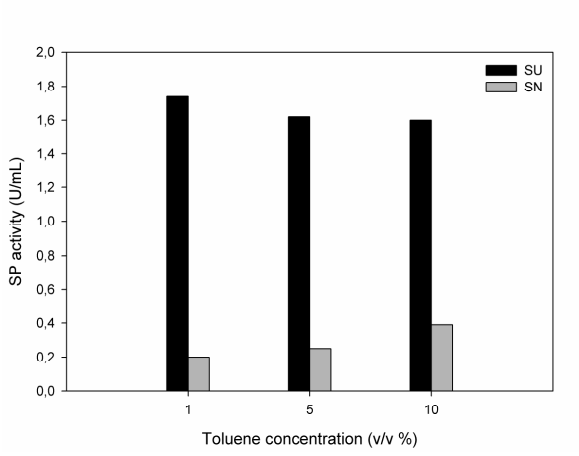

Figure 8.3 SP activity of cells treated for 5 min with 1 (v/v) % toluene.
The reference value was 2.25 U/mL.

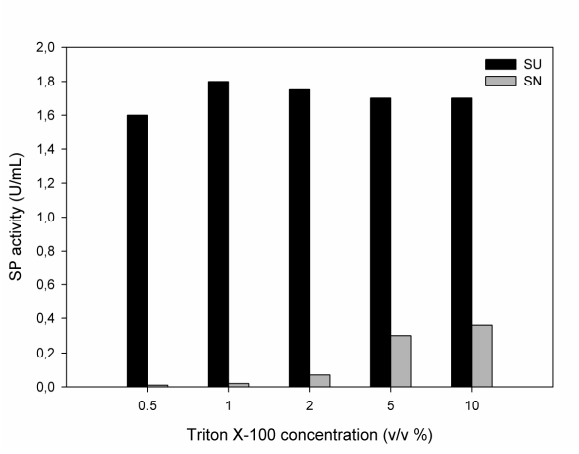

Figure 8.4 SP activity of cells treated for 5 min with 1 (v/v) % Triton X-100.
The reference value was 2.25 U/mL.

Figure 8.3 and Figure 8.4 show that increasing concentrations of toluene and Triton had almost no effect on the SP activity while they did have on cell leakage. Cells treated with only 1 (v/v) % toluene and Triton showed the highest SP activity with a maximum of 77 % and 80 %, respectively and also the lowest amount of leakage. At these conditions, 11 % of SP activity was found in the extracellular solution of the cells treated with toluene, while only 1 % was lost from the cells permeabilized with Triton. This indicates that the latter is less aggressive, leading to less damage to the cells. Higher concentrations of both agents resulted in SP leakages up to 24 % and 21 % for toluene and Triton, respectively. At concentrations above 10 (v/v) % toluene, a dramatically decrease in SP activity was observed, which might be attributed to the inactivation of sucrose phosphorylase by toluene.

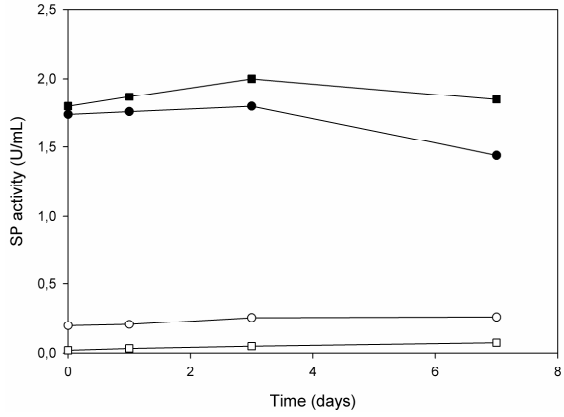

Figure 8.5 SP activity of cells treated with 1 (v/v) % toluene (●) and Triton X-100 (■). SP activity of their extracellular solution was also measured, shown as (○) and (□), respectively.

The *E. coli* cells treated with 1 (v/v) % toluene and Triton were then stored for 7 days at 4 °C. At certain times, SP activities of the treated cells as well as their extracellular solutions were measured to evaluate SP leaking. Figure 8.5 shows that only a small quantity of SP was lost (< 0.01 U/day) from both types of treated cells. After 3 days, the SP activity of the treated cells slightly increased. This is probably due to the fact that the leaked enzyme shows higher activity than the intracellular SP due to removal of diffusion limitations caused by the cell membrane. However, after 7 days storage at 4 °C, the activity of the cells again decreased. This might be caused by the fact that once the enzyme is removed from its cellular environment, it is less stable and denatures more easily. In conclusion, treatment with 1 (v/v) % toluene or Triton X-100 results in very similar effects, and both were used in further experiments.

In the previous tests, the *E. coli* cells were treated for 5 min with the different permeabilizers. In the next experiments, different treatment times were evaluated. Therefore, cells were treated on ice with 1 (v/v) % Triton X-100 for 0.5, 5, 10 and 15 min. After centrifugation, the cells were centrifugated and resuspended in 100 mM phosphate buffer, pH 7.0. Then, the treated cells, their extracellular solutions, and the permeabilization buffers were tested for SP activity (Figure 8.6). The crude SP preparation (reference value) had an SP activity of 2.55 U/mL.

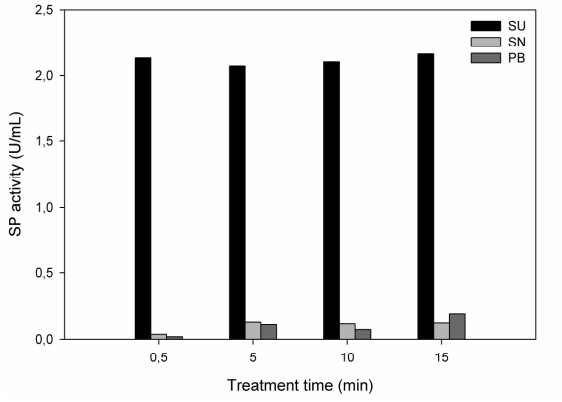

Figure 8.6 SP activity of SU, SN and PB after different treatment times with 1 (v/v) % Triton X-100. The reference value was 2.55 U/mL.

Figure 8.6 revealed that the different treatment times had little or no effect on the SP activity of the permeabilized cells with Triton. Compared to the crude SP preparation, relative SP activities of 81 % to 84 % were obtained. However, longer treatment resulted in increasing SP activities in the extracellular solution and permeabilization buffer, up to 12 % and 19 %, respectively. Therefore, the optimal condition seemed to be a treatment of 0.5 min with 1 (v/v) % Triton X-100. The same effect was observed for treatment with toluene. Higher treatment times had no effect on the activity of the permeabilized cells but resulted in more over-damaged cells and thus in higher cell leakage. Also for toluene, a treatment of 0.5 min gave the best results.

If we want to use these permeabilized cells for the production of αG1P, it is important that the cells can be used at temperatures above 4 °C. Therefore, as a last step, the storage temperature of the treated cells was evaluated. Cells treated with 1 (v/v) % toluene and Triton for 0.5 min were stored for 7 days at 4 °C and at room temperature. SP activities of the treated cells (SU) and their extracellular solution (SN) were measured (Figure 8.7 and Figure 8.8).

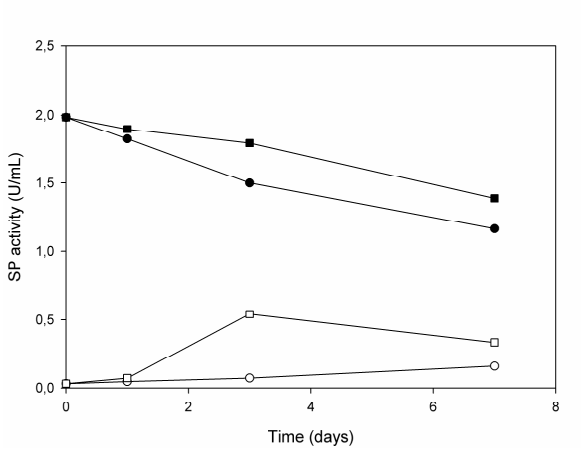

Figure 8.7 SP activities of cells stored at 4 °C or at room temperature. The cells were treated for 0.5 min with 1 (v/v) % toluene. SU (●) & SN (○) of cells stored at 4 °C, SU (■) & SN (□) of cells stored at room temperature.

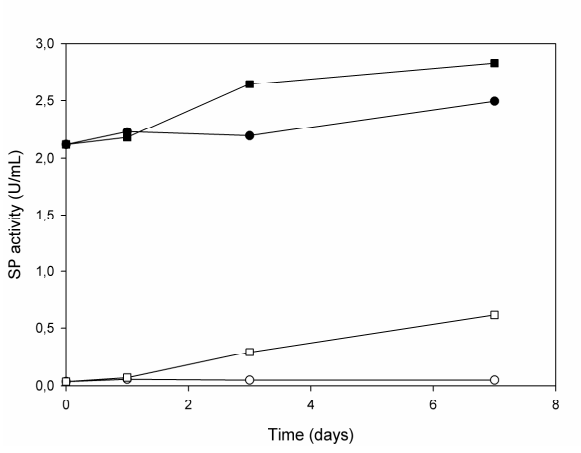

Figure 8.8 SP activities of cells stored at 4 °C or at room temperature. The cells were treated for 0.5 min with 1 (v/v) % Triton X-100. SU (●) & SN (○) of cells stored at 4 °C, SU (■) & SN (□) of cells stored at room temperature.

In general, when permeabilized cells were stored at higher temperatures, more cell leakage was observed. This was probably caused by small quantities of remaining permeabilizer in the cell membrane that further damage the cells at higher temperatures. Diffusion limitations due to the cell membrane diminished and the SP activity of the treated cells increased, as can be seen for

the cells treated with Triton. However, for cells permeabilized with toluene, a decrease in the SP activity was observed. This can be explained by the higher amount of SP leaking caused by toluene, as discussed previously. These extracellular SP molecules are less stable and denature more easily, especially at room temperature, which can be seen in the activity of the extracellular solution (SN) that decreased after 3 days storage at room temperature (Figure 8.7).

Based on these results, no production process for αG1P was performed. At room temperature, the treated cells were stable for only 1 day and from then on, cell leakage could not be neglected anymore. Increasing the temperature (for the production process of αG1P) would only enlarge the leaking problem. In addition, it would make no sense to recycle the permeabilized cells because they will lose a significant amount of enzyme during each production process.

3.5 Downstream processing of αG1P

After the continuous production of αG1P with immobilized SP in a packed bed reactor, the product was purified from the reaction mixture that also contains sucrose, phosphate, fructose and glucose (released by the hydrolytic activity of SP). The contaminating carbohydrates were removed by treatment with yeast (20 g/L). Instant baker's yeast was added to the mixture and the reaction was stirred for eight hours at 30 °C. Samples were taken and analyzed by HPLC (Figure 8.9).

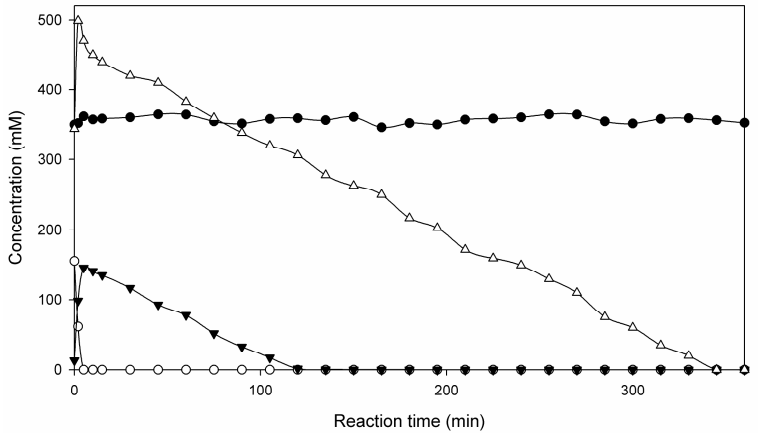

Figure 8.9 Removal of contaminating carbohydrates by treatment with yeast. Concentration of αG1P (●), fructose (Δ), glucose (▼) and sucrose (○) in function of the reaction time.

Initially, sucrose was converted to glucose and fructose by the yeast's invertase. This process was completed after 15 min. Meanwhile, glucose and fructose were metabolized and completely consumed within 2 and 6 hours, respectively. The αG1P, in contrast, was not consumed by the yeast and its concentration stayed constant during the treatment. Ethanol, CO_2 and some glycerol were formed during the reaction. The yeast was removed by filtration and αG1P was concentrated by evaporation at 60 °C to induce crystallization.

The end product, however, still contained remaining phosphate from the reaction mixture. This can be removed by chemical precipitation (Ashby *et al.*, 1955), but in that case crystals of αG1P could no longer be generated. This is probably caused by some of the components present in the precipitation liquid, *e.g.* acetate or Mg^{2+} ions. To solve this problem, the production process was adapted to minimize the amount of remaining phosphate. To that end, the phosphorolysis of sucrose was performed with varying concentrations of phosphate, allowing the reaction to proceed until apparent equilibrium has been attained (Figure 8.10). The assays were performed with purified SP and contained 500 mM sucrose. At equilibrium, αG1P, sucrose and phosphate concentrations were determined by HPLC (Table 8.2).

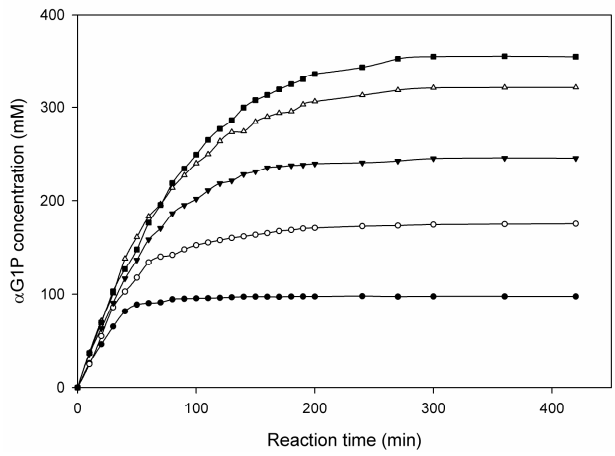

Figure 8.10 Production of αG1P from 500 mM sucrose and different concentrations of inorganic phosphate.
100 mM (●), 200 mM (○), 300 mM (▼), 400 mM (Δ) and 500 mM (■) phosphate buffer, pH 7.0.

Table 8.2 shows that lower phosphate concentrations at the start of the reaction result in lower phosphate concentrations at equilibrium. However, the degree of conversion is also lower in that case, which compromises the yield of αG1P. The optimal sucrose/phosphate ratio will thus depend on the specifications of the end-product. A ratio of 5 will guarantee low phosphate concentrations in the product and facilitate the downstream processing.

Table 8.2 Phosphorolysis of 500 mM sucrose with varying concentrations of phosphate

[Phosphate][a] (mM)	[αG1P][b] (mM)	[Phosphate][b] (mM)	Conversion (%)
100	97.6	2.4	19.5
200	175.1	25.0	35.0
300	245.5	54.6	49.1
400	321.1	78.9	64.2
500	354.5	145.6	70.9

[a] Concentration before the reaction; [b] Concentration at equilibrium.

4. CONCLUSION

In this chapter three different strategies were described for the production of αG1P by sucrose phosphorolysis, each having pros and cons. When using SP from *B. adolescentis* covalently bound on Sepabeads, a continuous production of αG1P in a packed bed reactor at 60 °C could be performed for more than 10 days without loss of activity. In turn, cross-linked enzyme aggregates of SP could be used for seven successive batch reactions 60 °C without loss of activity. Both immobilization techniques have the advantage that they allow easy reuse and recovery from the reaction mixture.

Whole-cell bioconversions typically are very practical and cost-effective solutions for industrial applications. Unfortunately, they were found to be less suited for the phosphorolysis of sucrose. Indeed, cells had to be permeabilized to allow diffusion of substrate and product through the cellular membrane, which resulted in too much leakage of SP enzyme from the cells.

In conclusion, this is the first time that a production process for αG1P with the SP from *B. adolescentis* is described at elevated temperatures, either as cross-linked enzyme aggregates or immobilized on Sepabeads EC-HFA. The theoretical maximal yield of 72 % could be easily obtained and the product could be conveniently purified by removing contaminating carbohydrates by treatment with yeast.

CHAPTER 9

GENERAL DISCUSSION

1. INTRODUCTION

The transfer of a glycosyl group is one of the most important biochemical reactions in nature and also has a wide range of industrial applications (Sinnott, 1994). Oligosaccharides, for example, have great potential in the food industry, not only as essential nutrients that stimulate the immune system but also as low-caloric and non-cariogenic sweeteners. In turn, glycosylation of a non-carbohydrate acceptor, resulting in a glycoside or a glycoconjugate, can drastically change both the physicochemical and biological properties of that molecule (Kren, 2008). Attaching a glycosyl group to a vitamin, for example, can improve its stability, solubility and bio-availability.

As carbohydrates can be branched and connected in many different ways due the presence of multiple hydroxyl groups, their potential structural diversity is enormous. Consequently, **chemical synthesis of glycosidic molecules** is a very challenging task that requires the use of protecting and activating groups, resulting in multi-step synthetic routes with a low overall yield. Furthermore, chemical synthesis also makes use of toxic catalysts such as heavy metals, which limits its application in large-scale processes. Therefore, enzymatic glycosylation reactions have attracted increasing attention as a solution to overcome these obstacles. Indeed, enzymes are highly specific and are active under mild conditions (de Roode *et al.*, 2003). They typically have a space-time yield that is 15 times higher than chemical synthesis and generate 5 times less waste.

Processing of glycosidic molecules involves a wide range of enzymes. Among them, glycoside phosphorylases have some interesting properties (Kitaoka & Hayashi, 2002). For example, their glycosyl donor -a glycosyl phosphate- is much cheaper and available in larger quantities than the nucleotide-activated donors of glycosyl transferases. In comparison with glycoside hydrolases, their reactions show a higher degree of regioselectivity and have a substantially higher yield. We have selected **sucrose phosphorylase as biocatalyst** for glycosylation reactions because it can transfer the glucosyl moiety of sucrose to a wide variety of acceptor molecules. Unfortunately, the thermostability of this enzyme is too low for industrial applications, which need to be operated at 60 °C or higher to avoid microbial contamination (Vieille & Zeikus, 2001). In this work, various strategies will be evaluated to improve the performance of sucrose phosphorylase at elevated temperatures.

In summary, the major achievements of this PhD thesis are:

1) **The construction of an inducible and constitutive expression system for SP.** The expression of SP was optimized for both systems and similar activity levels were obtained (CHAPTER 2).

2) **The development of a new assay for the determination of SP activity.** This method measures the release of the reducing sugar fructose from the non-reducing substrate sucrose and is optimally suited for screening purposes (CHAPTER 2).

3) **The characterization of the SP enzymes from *L. mesenteroides* and *B. adolescentis*.** The latter was found to be considerably more stable and to have a temperature optimum that is 16 °C higher (CHAPTER 2).

4) **The identification of determinants of SP thermostability.** Based on sequence alignment and mutational analysis, two amino acid substitutions have been identified that have a rigidifying effect on the enzyme's structure (CHAPTER 3).

5) **The optimization of a high throughput screening procedure for thermostability.** Inactivation in PCR plates was found to be crucial to lower the variation (CV) down to 15 % (CHAPTER 4).

6) **The creation of SP enzyme variants with enhanced thermostability.** By means of (semi-) rational design, five mutants have been generated that are about 40 % more stable than the wild-type enzyme (CHAPTER 5).

7) **The immobilization of SP by multipoint covalent coupling to a Sepabeads carrier.** The obtained biocatalyst is exceptionally stable at 60 °C and displays an increased temperature optimum (CHAPTER 6).

8) **The immobilization of SP in the form of cross-linked enzyme aggregates.** The obtained biocatalyst is exceptionally stable at 60 °C and displays an increased temperature optimum (CHAPTER 7).

9) **The production and purification of α-D-glucose-1-phosphate.** For the first time, a production process with SP has been developed at elevated temperatures, serving as proof of concept for the industrial application of the enzyme (CHAPTER 8).

2. CONCLUSIONS

To date, sucrose phosphorylase (SP) has been identified in only a relatively small number of bacterial species, none of which are thermophilic. As carbohydrate conversions need to be operated at 60 °C or higher to avoid microbial contamination, the lack of a thermostable enzyme is a major limitation of the industrial application of SP. Consequently, the goal of this PhD thesis is to **increase its thermostability by various engineering strategies**. To that end, the most promising SP enzymes have first been recombinantly expressed and thoroughly characterized.

Two sucrose phosphorylases have been selected as template for engineering, *i.e.* that from *L. mesenteroides* (*Lm*SP) and that from *B. adolescentis* (*Ba*SP). The former was chosen because it has been used for the majority of reported glycosylation reactions (Goedl *et al.*, 2010) and because our laboratory has acquired extensive expertise with it (Vandamme *et al.*, 1987). The latter was chosen because its crystal structure is available (Sprogoe *et al.*, 2004) and because it exhibits the highest temperature optimum reported so far (van den Broek *et al.*, 2004).

To facilitate the high-throughput screening of SP, a constitutive expression system has been evaluated as an alternative to classical inductive systems (CHAPTER 2). In that way, the induction step can be circumvented, which should drastically increase the throughput and accuracy of the process. Our results show that **the constitutive expression of SP yields** about the same activity level as inductive expression, *i.e.* about 1 U/mL. Furthermore, a new assay has been developed for SP, which is optimally suited for screening purposes. It is based on the detection of the reducing sugar fructose from the non-reducing substrate sucrose.

The characterization of the SP from *B. adolescentis* has revealed that this enzyme exhibits a relatively high temperature optimum (58 °C) and **a remarkable stability at 60 °C** (CHAPTER 2). In contrast, the SP from *L. mesenteroides* has an optimal temperature of only 42 °C and loses all of its activity after 5 minutes incubation at 60 °C. Interestingly, the thermostability of purified SP was found to depend strongly on the enzyme concentration, with higher concentrations being considerably more stable. Although the effect is somewhat less pronounced in crude enzyme preparations, this phenomenon caused some serious problems during the screening of SP variants with improved thermostability.

The intriguing difference in thermostability of the SP enzymes from *B. adolescentis* and *L. mesenteroides* has been examined in more detail by means of mutational analysis (CHAPTER 3). Based on a sequence alignment with *Lm*SP, 20 amino acid residues were selected that could possibly contribute to the high stability of *Ba*SP. However, only two of these turned out to be

actual **determinants of thermostability**. More specifically, introducing prolines at positions 139 and 162 of *Lm*SP generates enzyme variants that are 14 and 21 %, respectively, more stable than the wild-type enzyme. Combining these mutations with the previously identified mutation V131L (Fujii *et al.*, 2006) results in an overall increase of 40 %.

Further engineering of thermostability was performed with the SP from *B. adolescentis*, as this provides a template that is already more stable to begin with. An **automated high-throughput screening** procedure was first optimized with the wild-type enzyme. Inactivation in PCR plates was found to be crucial to lower the variation (CV) down to 15 % (CHAPTER 4). In an approach known as B-FIT (Reetz *et al.*, 2006), several residues with high flexibility (high B-factors) were then selected for site-saturation mutagenesis. Unfortunately, the screening of the mutant libraries was plagued by false positives, due to variations in expression levels that result in an overestimation of the enzymes' thermostability. The latter is a peculiar characteristic of SP and is not typically observed with other enzymes. Indeed, it has been possible to improve the thermostability of many enzymes by high throughput screening of mutant libraries (Eijsink *et al.*, 2005).

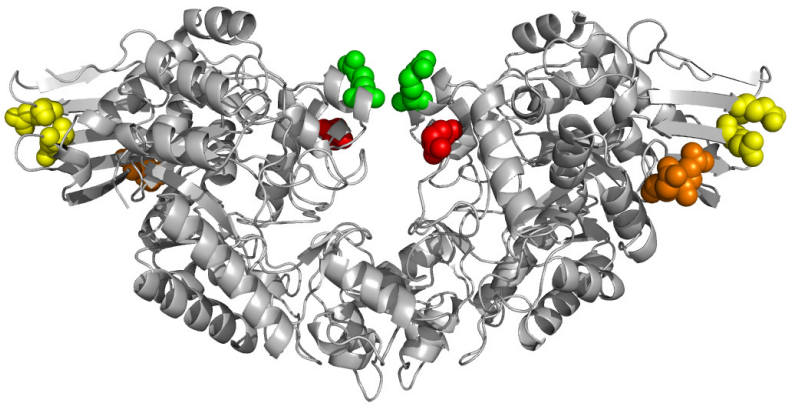

Figure 9.1 The sites in SP from *B. adolescentis* where mutations have a positive effect on the thermostability.
Sites Q331 (red), R393 (green), D445/D446 (yellow) and Q460/E485 (orange).

To circumvent this problem, rational input has been used to limit the size of the libraries, allowing the individual processing of the enzyme variants. In that way, site-directed mutants can be diluted manually to the same level of activity, effectively eliminating the influence of concentration on the enzyme's stability. Rational input was obtained from the 3DM-database that contains structure-based sequence alignments of protein families (Kuipers *et al.*, 2010). The

frequency of amino acids is calculated at each structurally conserved position, information that was used to limit the randomization in the B-fit libraries to those residues that are known to be favoured by nature. In total, only eight mutants now had to be screened, of which **two displayed a considerable increase in thermostability** (CHAPTER 5, Figure 9.1). Indeed, the substitution of the DD-motif at positions 445 and 446 of *Ba*SP with PT and PG resulted in a relative stability of 137 % and 139 %, respectively, compared to the wild-type enzyme (Figure 9.2). The corresponding variants were then purified to characterize their thermostability in more detail. Incubating the enzymes for one hour at different temperatures revealed that their T_{50}^{60} value, the temperature at which half of the activity has been lost, is about 66 °C, which represents an increase of approximately 1 °C compared to that of the wild-type enzyme.

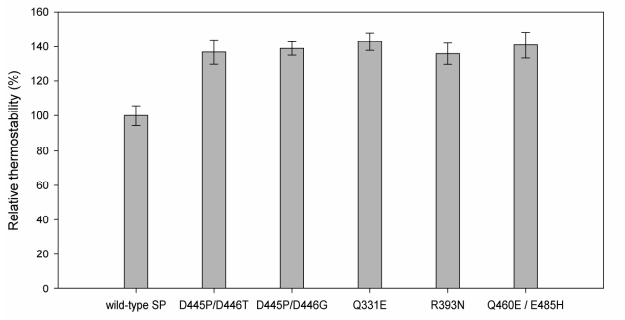

Figure 9.2 Relative thermostability after 15 min incubation at 70 °C of all improved *Ba*SP variants.
Wild-type *Ba*SP, with a residual activity of 30 ± 1.7 %, is used as reference.

Alternatively, rational input was obtained by inspecting the enzyme's crystal structure. Although there still are no general rules to stabilize 'any' protein by a limited number of specific mutations, some trends have emerged (Eijsink *et al.*, 2004). Therefore, the structure of *Ba*SP was examined for amino acid substitutions that might result in additional salt bridges, increased interactions at the dimer interface or stabilization of helix dipoles. In total, twelve mutants were screened, of which **three displayed a considerable increase in thermostability** (CHAPTER 5, Figure 9.1). Indeed, variants Q331E, R393N and Q460E/E485H have a relative stability of 143, 136 and 141 %, respectively, compared to the wild-type enzyme (Figure 9.2). Characterization of the purified enzymes revealed that their T_{50}^{60} value is about 1 °C higher than that of the wild-type enzyme. Furthermore, combining two of these mutations was found to have an additive effect, *i.e.* resulting in an increase of 2 °C.

Although several variants of *Ba*SP with improved thermostability are now available, their performance is still not adequate for industrial applications. Therefore, **multi-point covalent immobilization** of the enzyme has been evaluated as an alternative strategy, which would have the additional advantage that the biocatalyst becomes recyclable for repetitive use or for application in a continuous production process. Optimization of the immobilization on Sepabeads EC-HFA by means of experimental design, resulted in an activity yield of 72 % (CHAPTER 6). Furthermore, the temperature optimum for activity was found to have shifted from 58 °C to 65 °C (Figure 9.3), and both the thermal and pH stability were considerably enhanced. The obtained biocatalyst should thus be useful for application in carbohydrate conversions at high temperature. The only downside of this procedure is the high price of the carrier, which could become a major issue at the industrial scale.

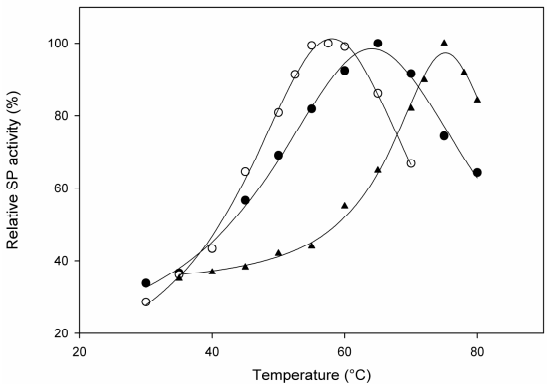

Figure 9.3 Thermoactivity of free *Ba*SP (○) and immobilized *Ba*SP on Sepabeads EC-HFA (●) and as CLEAs (▲). Reactions were performed with 100 mM sucrose in a 100 mM phosphate buffer at pH 7.0.

To avoid the use of an expensive carrier, the enzyme has also been immobilised by the production of **Cross-Linked Enzyme Aggregates** (CLEAs). The cost has been lowered even further by using heat-purified enzyme, circumventing the chromatographic separation step based on the affinity of a His-tag. Optimization of the immobilization procedure resulted in an activity yield of 31 % (CHAPTER 7). The temperature optimum of the obtained enzyme preparation was found to be increased by as much as 17 °C (Figure 9.3), while the pH profile had significantly broadened. Furthermore, the CLEAs remain fully active after one week incubation at 60 °C, paving the way for their use in successive batch reactions. This low-cost and simple procedure should allow the development of industrial processes to exploit the various glycosylation reactions catalyzed by sucrose phosphorylase.

Finally, the performance of the different types of immobilized enzymes has been **evaluated in a production process** for α-D-glucose-1-phosphate (αG1P) from the cheap substrate sucrose at a temperature of 60 °C (CHAPTER 8). With SP from *B. adolescentis* covalently bound to Sepabeads, a continuous production process in a fixed-bed reactor could be performed for more than 10 days without loss of activity. The theoretical maximal yield of 71 % was readily obtained, and the product could simply be purified by removing the contaminating sugars by treatment with baker's yeast. In turn, CLEAs of SP could be recycled for at least ten successive batch reactions, yielding more than 1 kg of product at a very attractive price.

In addition, **a whole-cell bioconversion** has also been evaluated with a recombinant *E. coli* that expresses sucrose phosphorylase. In that case, the enzyme from *L. mesenteroides* has been used as biocatalyst because its temperature optimum corresponds better with that of the host cell. Permeabilization of the cell membrane with either toluene or Triton X-100 proved to be necessary to allow diffusion of substrate and product in and out the cell (CHAPTER 8). Unfortunately, this also resulted in considerable leakage of the enzyme, which means that recycling of the cells becomes rather inefficient. Although the enzyme seems to be more stable inside than outside the cell, this strategy is less efficient than the other techniques described in this thesis and has, therefore, been abandoned.

In conclusion, **several strategies have been successfully applied** in this work to increase the thermostability of sucrose phosphorylase. Engineering of the enzyme by (semi-)rational mutagenesis has resulted in a number of variants with improved properties that could potentially be combined to obtain a stable biocatalyst at 60 °C. However, immobilization of the enzyme, either by covalent attachment to a carrier or by cross-linking, was found to be a more efficient technique, as it generates a biocatalyst that is stable for at least 2 weeks at 60 °C and can be used for more than one reaction cycle. For the first time, production of αG1P has become possible at elevated temperatures, which serves as proof of concept for the production of other glycosylated compounds with SP under industrial conditions.

3. PERSPECTIVES

The site-directed mutants created in this study only display a moderate increase in thermostability (CHAPTER 5). Although combining all of them in a single sequence might generate an enzyme variant that is considerably more stable, the success rate of our rational design was rather low. This could mean that the selected residues are not located in the structural region that is most sensitive to unfolding. Indeed, it has been shown that thermal inactivation can be governed by local unfolding processes that involve only a small part of the protein (Eijsink *et al.*, 2001). Consequently, not all mutations that increase the thermodynamic stability (reversible unfolding) will have a similar effect on the kinetic stability (irreversible inactivation).

If the thermal inactivation of sucrose phosphorylase is indeed governed by local unfolding, the key to a successful stabilization would be the elucidation of the mechanism of denaturation. This is, however, not an easy task. One of the most informative analyses for the study of protein folding is circular dichroism (CD), which measures changes in secondary structures (Cai & Dass, 2003). Unfortunately, the exact position of these structures within the general fold cannot be determined. Alternatively, molecular dynamics simulations can be an important tool as it allows to study the unfolding of a protein *in silico* (Scheraga *et al.*, 2007). Identifying the regions of the protein that unfold first will undoubtedly increase the efficiency of engineering of SP towards increased thermostability.

Since the SP from *B. adolescentis* forms a dimer, one of the most crucial regions for its stability could well be the subunit interface. Indeed, subunit interactions have been described as one of the major stabilization forces in thermophilic proteins and an ever-increasing number of thermophilic proteins are known that have a higher oligomerization state than their mesophilic homologues (Mrabet *et al.*, 1992; Vieille & Zeikus, 2001). In fact, this could also be the reason why the SP from *B. adolescentis* is much more stable than its mesophilic counterpart from *L. mesenteroides*, which is a monomer.

In addition to the study of the unfolding mechanism of SP, several other suggestions can be formulated for further experiments based on the results described in this thesis.

- **The determination of the crystal structure of SP from *L. mesenteroides*.** To date, structural information is only available for the SP enzyme from *B. adolescentis*, which is a dimer. The elucidation of the 3D structure of *Lm*SP, which is a monomer, would give new insights in the determinants of thermostability of SP enzymes. It can be expected that the latter enzyme employs a different mechanism for stabilization than *Ba*SP, which will

generate new ideas for engineering. Compared to the SP from *B. adolescentis*, other residues might, for example, have a high degree of flexibility (high B-factor) and thus form targets for randomization.

- **Co-expression of molecular chaperones to facilitate the screening of mutant libraries.** Directed evolution of the thermostability of SP has proven to be very challenging because the produced enzymes variants show a considerable variation in expression level. This results in the detection of false positives during the screening, as thermostability is over-estimated at low protein concentrations. One of the solutions for this problem could be the co-expression of molecular chaperones that assist in protein folding and maximize the amount of soluble enzyme (Tokuriki & Tawfik, 2009a).

- **Determining the solvent stability of SP variants.** Glycosylation reactions of hydrophobic acceptors need to be performed in the presence of organic co-solvents in order to increase their solubility. As enzymes with improved thermal stability usually also become more resistant to other denaturing factors (Eijsink *et al.*, 2004), it would be very interesting to screen the improved SP variants described in this thesis for stability against various solvents.

- **Determining the acceptor specificity of immobilized SP.** The immobilized biocatalyst exhibited a slightly higher K_M for its natural substrate than the soluble enzyme (CHAPTER 6). Changes in substrate affinity could be caused by distortion of the active site during the immobilization process. It is, however, not clear if a similar effect would be observed for other glycosyl acceptors as well. For example, Hormigo and coworkers reported that the immobilization of penicillin G acylase on Sepabeads increases its affinity for non-natural substrates like penicillin K (Hormigo *et al.*, 2009). It would, therefore, be very interesting to examine the substrate specificity of immobilized SP in more detail.

- **Engineering the acceptor specificity of SP.** To exploit the various glycosylation reactions catalyzed by SP at the industrial scale, its activity towards non-natural acceptors will have to be increased by means of enzyme engineering. It has been shown that stable proteins are better templates for engineering purposes because they can withstand the destabilizing effects of introduced mutations (Tokuriki & Tawfik, 2009b). The improved enzyme variants described in this thesis could thus constitute a very valuable starting point to develop a platform technology for the glycosylation of small molecules with sucrose phosphorylase.

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SUMMARY

SAMENVATTING

SUMMARY

The synthesis of glycosidic bonds is of high commercial value, because the produced compounds can be used for a wide range of applications. Oligosaccharides, for example, have great potential in the food industry, not only as essential nutrients that stimulate the immune system but also as low-caloric and non-cariogenic sweeteners. In turn, glycosylation of a non-carbohydrate acceptor, resulting in a glycoside or a glycoconjugate, can drastically influence both the physicochemical and biological properties of that molecule. Attaching a glycosyl group to a vitamin, for example, can improve its stability, solubility and bio-availability.

As carbohydrates can be branched and connected in many different ways due the presence of multiple hydroxyl groups, their potential structural diversity is enormous. Consequently, chemical synthesis of glycosidic molecules is a very challenging task that requires the use of protecting and activating groups, resulting in multi-step synthetic routes with a low overall yield. Furthermore, chemical synthesis also makes use of toxic catalysts such as heavy metals, which limits its application in large-scale processes. Enzymatic glycosylation methods are therefore preferred since they result in higher yields and are more regiospecific than the chemical methods.

Many enzymes can be applied for the production of glycosides. We have selected sucrose phosphorylase (SP) for glycosylation reactions because it can transfer a glucosyl moiety from an inexpensive donor substrate -simple table sugar- to a wide variety of acceptor molecules. Unfortunately, the thermostability of this enzyme is too low for industrial applications, which need to be operated at 60 °C or higher to avoid microbial contamination. Consequently, the goal of this PhD thesis is to increase the thermostability of sucrose phosphorylase.

First, the most promising SP enzymes, *i.e.* from *L. mesenteroides* (*Lm*SP) and *B. adolescentis* (*Ba*SP) were recombinantly expressed and thoroughly characterized. The characterization of *Ba*SP has revealed that this enzyme exhibits a relatively high temperature optimum (58 °C) and a remarkable stability at 60 °C. In contrast, *Lm*SP has an optimal temperature of only 42 °C and loses all of its activity after 5 minutes incubation at 60 °C. The intriguing difference in thermostability of these two SP enzymes has been examined in more detail. Based on sequence alignment and mutational analysis, two amino acid substitutions have been identified that have a rigidifying effect on the enzyme's structure.

Several strategies have then been successfully applied to increase the thermostability of SP from *B. adolescentis*. Engineering of the enzyme by (semi-)rational mutagenesis has resulted in five mutants that are about 40 % more stable than the wild-type enzyme. These beneficial mutations

could potentially be combined to obtain a stable biocatalyst at 60 °C. However, immobilization of the enzyme, either by covalent attachment to a carrier or by cross-linking, was found to be a more efficient technique, as it generates a biocatalyst that is stable for at least 2 weeks at 60 °C and can be used for more than one reaction cycle. Furthermore, the temperature optimum of the immobilized enzyme was found to be increased by as much as 17 °C, in the case of the cross-linked enzyme. For the first time, production of αG1P has become possible at elevated temperatures, which serves as proof of concept for the production of other glycosylated compounds with SP under industrial conditions.

SAMENVATTING

De transfer van een glycosyl groep is één van de belangrijkste biochemische reacties in de natuur, en heeft ook een brede waaier aan toepassingsmogelijkheden. Oligosacchariden, bijvoorbeeld, kregen de laatste jaren veel aandacht, niet alleen door hun potentiële prebiotische effecten in voedingspreparaten maar ook als laag calorische en niet-cariogene zoetstof. Daarnaast kan het glycosyleren van niet-suiker verbindingen, resulterend in een glycoside of een glycoconjugaat, een sterke invloed hebben op zowel de fysicochemische als de biologische eigenschappen van de molecule. Het binden van een koolhydraatgroep aan een vitamine, bijvoorbeeld, kan de stabiliteit, oplosbaarheid en bio-beschikbaarheid positief beïnvloeden.

De structurele diversiteit van suikers is enorm daar ze op zeer veel verschillende manieren vertakt en verbonden kunnen worden door de aanwezigheid van de vele hydroxyl groepen. Bijgevolg is de chemische synthese van glycosidische moleculen een zeer grote uitdaging die meestal het gebruik van protectie en activatie groepen vereist, wat resulteert in meerstaps reacties met een lage opbrengst. Daarenboven worden er vaak toxische katalysatoren, zoals zware metalen, gebruikt hetgeen grootschalige toepassingen verder belemmert. Hierdoor worden enzymatische glycosylatiemethoden meestal verkozen boven de chemische methoden, aangezien ze resulteren in hogere opbrengsten en meer regiospecifiek zijn.

Er zijn veel enzymen die kunnen toegepast worden voor de productie van glycosiden. In dit doctoraatsonderzoek werd sucrose phosphorylase (SP) geselecteerd voor glycosyleringsreacties, omdat het een glucosyl groep transfereert van een goedkoop donor substraat -eenvoudig tafelsuiker- naar een brede waaier van acceptors. Helaas is de thermostabiliteit van dit enzym te laag voor industriële toepassingen, die preferentieel uitgevoerd worden bij 60 °C of hoger, hoofdzakelijk om microbiële contaminatie tegen te gaan. Bijgevolg is het doel van deze doctoraatsthesis de thermostabiliteit van sucrose phosphorylase te verhogen.

Eerst werden de meest belovende SP enzymen, nl. van *L. mesenteroides* (*Lm*SP) en van *B. adolescentis* (*Ba*SP), recombinant tot expressie gebracht en grondig gekarakteriseerd. De karakterisatie van *Ba*SP onthulde dat dit enzym een relatief hoge optimale temperatuur vertoont (58 °C) en een opmerkelijke stabiliteit bij 60 °C. *Lm*SP heeft daarentegen een optimale temperatuur van slechts 42 °C en verliest al zijn activiteit na 5 minuten incubatie bij 60 °C. Dit intrigerend verschil in thermostabiliteit werd dan ook in meer detail bestudeerd. Via sequentie-alignering en mutationeel onderzoek werden twee aminozuur substituties geïdentificeerd die een stabilizerend effect hebben op de structuur van het enzym.

In een tweede fase werden verschillende strategiën met succes toegepast om de thermostabiliteit van SP van *B. adolescentis* te verhogen. Engineering van het enzym via (semi-)rationele mutagenese resulteerde in 5 varianten die ongeveer 40 % meer stabiel zijn dan het wild-type enzyme. Deze mutaties zouden gecombineerd kunnen worden om een stabiele biokatalysator bij 60 °C te bekomen. Immobiliseren van het enzym via covalente binding op een carrier of via cross-linking bleek evenwel een meer efficiente techniek te zijn. Het geïmmobiliseerde enzym is voor minstens 2 weken stabiel en kan gebruikt worden in meer dan 1 reactie cyclus. Daarenboven vertoont het gecrosslinkt enzym een stijging in optimale temperatuur van niet minder dan 17 °C. Het is de eerste keer dat de productie van αG1P bij verhoogde temperaturen mogelijk wordt en dit dient als *proof of concept* voor de productie van andere geglycosyleerde verbindingen met sucrose phosphorylase bij industriële condities.

CURRICULUM VITAE

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1. PERSONALIA

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Belgian nationality

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2. ACADEMIC EDUCATION

2010	**Doctor of Bioscience Engineering, Ghent University** thesis: 'Increasing the thermostability of sucrose phosphorylase' Department of Biochemical and Microbial Technology, Laboratory for Industrial Biotechnology and Biocatalysis, Faculty of Bioscience Engineering Em. Prof. dr. ir. E. J. Vandamme and Prof. dr. ir. W. Soetaert
2003	**Master of Bioscience Engineering (chemistry), Ghent University** thesis: 'Determination of minimal residual disease in neuroblastoma patients with quantitative RT-PCR' Department of Pediatric Hematology and Oncology, Ghent University Hospital Prof. dr. J. Philippé & Prof. dr. P. Van Oostveldt
1998	**Secondary School: Latin-Science** (Bisschoppelijk College, Veurne)

3. SUPPLEMENTARY EDUCATION

2008	Training for Teaching Assistants (3 days): Ghent University
2008	Doctoral training program: Ghent University
2004	Summer Course 'Glycosciences' (4 days, Wageningen, The Netherlands)
2004	Software for Fermentation Control (1 day, Sartorius, Belgium)

3. PROFESSIONAL ACTIVITIES

Dec 2003 – Nov 2010 **PhD student / Teaching assistant (AAP)** at the Laboratory for Industrial Biotechnology and Biocatalysis, Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University.

Support of theoretical and responsible for practical courses:
- Microbiology
- Biocatalysis and enzyme technology
- Industrial microbiology and biotechnology
- Industrial fermentation processes and downstream processing

Promotor of Master students:
- De Winter Karel (2009-2010) Increasing the thermostability of sucrose phosphorylase by covalent immobilization.
- Callens Evelien (2008-2009) Immobilization of disaccharide phosphorylases.
- Cools Ben (2008-2009) Biocatalytic conversion of sucrose to cellobiose.
- Moreira Carolina (2007-2008) Production of a thermostable sucrose phosphorylase.
- Aerts Dirk (2006-2007) Recombinant overexpression of cellobiose phosphorylase and sucrose phosphorylase in *Escherichia coli*.
- De Clercq Eveline (2006-2007) Cloning and gene expression of new cellobiose phosphorylase genes.
- Van Gastel Nick (2006-2007) Cloning and characterization of cellobiose phosphorylases.
- Gabriela Martins (2005-2006) Identification of a novel cellobiose phosphorylase in *Cellulomonas fimi*.
- Teresa Sanchez-Tejerina (2005-2006) Recombinant expression of cellobiose phosphorylase and sucrose phosphorylase in *Escherichia coli*.

4. PUBLICATIONS

- Naessens, M., Cerdobbel, A., Soetaert, W. & Vandamme, E. J. (2005) Dextran dextrinase and dextran of *Gluconobacter oxydans*. Journal of Industrial Microbiology and Biotechnology, 32, 323-334.
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- Cerdobbel, A., Desmet, T., De Winter, K. & Soetaert, W. (2010) Sucrose phosphorylase as cross-linked enzyme aggregate: Improved thermal stability for industrial applications. Biotechnology Journal, 5, 1192-1197.
- Cerdobbel, A., Desmet, T. & Soetaert, W. (2010) Increasing the thermostability of sucrose phosphorylase by rational and random mutagenesis (in preparation).

5. PATENTS

- Cerdobbel, A., Desmet, T. & Soetaert, W. (2010). A thermostable sucrose phosphorylase. UK priority filing 1005754.5

6. CONFERENCES

- 2009, June 10-12 (Ghent, Belgium), 5th International Conference on Renewable Resources and Biorefineries (RRB5)
- 2009, May 10 – 13 (Ischia, Italy), 8th Carbohydrate Bioengineering Meeting (CBM8)
- 2008, October 25 – 30 (San Feliu de Guixols, Spain), Protein Design and Evolution for Biocatalysis
- 2007, June 4 – 6 (Ghent, Belgium), 3th International Conference on Renewable Resources and Biorefineries (member of the Organisation Committee)
- 2007, April 22 – 25 (Braunschweig, Germany), 7th Carbohydrate Bioengineering Meeting (CBM7)
- 2005, October 6 (Leuven, Belgium), 11th Symposium on Applied Biological Sciences (PhD Symposium)
- 2005, September 19 – 21 (Ghent, Belgium), International Conference on Renewable Resources and Biorefineries (member of the Organisation Committee)
- 2005, April 3 – 6 (Barcelona, Spain), 6th Carbohydrate Bioengineering Meeting (CBM6)
- 2006, May 17 – 18 (Rennes, France), Symposium of Enzymes for Food
- 2004, January 15 (Brussel, Belgium), Industrial Biotechnology and Sustainable Chemistry: Perspectives and Policy Issues (member of the Organisation Committee)

7. SERVICES

- Organization of the Annual Job Fare for Bio-engineers (2006-2010)
- Secretary of the Committee for Environment, Safety and Hygiene (2007-2010)
- Member of the Committee for Environment, Safety and Hygiene (2005-2007)
- Member of the Departmental Council (2005-2010)
- Member of the Building Committee (2005-2010)
- Member of the Committee for the promotion of Tenured Academic Staff (ZAP) (2008)
- Contribution to 'Vlaamse Wetenschapsweek', Scientists@work, information days at Faculty of Bioscience Engineering, presentations at secondary schools, ...
- Writing research proposals for IWT, FWO and BOF

APPENDICES

APPENDIX I. THE STANDARD GENETIC CODE

1st position (5' end)	2nd position U	2nd position C	2nd position A	2nd position G	3rd position (3' end)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

APPENDIX II. NUCLEOTIDES AND THEIR ABBREVIATIONS (IUB CODE)

Nucleotide	Name	Complement
A	Adenine	**T**
C	Cytosine	**G**
G	Guanine	**C**
T	Thymine	**A**
U	Uracil (RNA)	**A**
R	puRine (G of A)	**Y**
Y	pYrimidine (C or T)	**R**
W	Weak (A or T)	**W**
S	Strong (G or C)	**S**
M	aMino (A or C)	**K**
K	Keto (G or T)	**M**
V	not T (A, C or G)	**B**
B	not A (C, G or T)	**V**
H	not G (A, C or T)	**D**
D	not C (A, G or T)	**H**
N	A, G, C or T	**N**

APPENDIX III. AMINO ACID TERMINOLOGY

Amino acid	3-letter code	1-letter code
Alanine	Ala	**A**
Arginine	Arg	**R**
Asparagine	Asn	**N**
Aspartic acid	Asp	**D**
Cysteine	Cys	**C**
Glutamic acid	Glu	**E**
Glutamine	Gln	**Q**
Glycine	Gly	**G**
Histidine	His	**H**
Isoleucine	Ile	**I**
Leucine	Leu	**L**
Lysine	Lys	**K**
Methionine	Met	**M**
Phenylalanine	Phe	**F**
Proline	Pro	**P**
Serine	Ser	**S**
Threonine	Thr	**T**
Tryptophan	Trp	**W**
Tyrosine	Tyr	**Y**
Valine	Val	**V**